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Cover photo:
Gems and Arabidopsis

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Memoria presentada por Elena Caro Bernat, Licenciada en Bioquímica, para optar al grado de Doctor en Ciencias por la Universidad Autónoma de Madrid.

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CENTRO DE BIOLOGÍA MOLECULAR "SEVERO OCHOA"

A mis padres, mi Tate
y mi Ángel de la guarda.

The sweetest and most inoffensive path of life leads through the avenues of science and learning; and whoever can either remove any obstructions in this way, or open up any new prospect, ought so far to be esteemed a benefactor to mankind.

David Hume (1711–1776), filósofo escocés.
En "An Enquiry Concerning Human Understanding"

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Resumen

El desarrollo de los organismos multicelulares complejos requiere la precisa especificación de diversos tipos celulares en momentos y localizaciones específicas. La coordinación entre la especificación de la identidad celular y la progresión a lo largo del ciclo celular es necesaria para un asegurar un correcto desarrollo.

En la epidermis de la raíz de *Arabidopsis thaliana*, la expresión del gen homeobox *GLABRA2* (*GL2*) determina la identidad celular pelo/no pelo radicular. Hemos identificado una nueva proteína, GEM (*GL2* expression modulator), que interacciona con los dos homólogos de Cdt1 en Arabidopsis y que regula el patrón epidérmico de la raíz durante su desarrollo. GEM interacciona con TTG1 (TRANSPARENT TESTA GLABRA 1), una proteína de repeticiones WD40 involucrada en la toma de decisiones de identidad que modula tanto la división celular como la expresión de *GL2*. Nuestros estudios revelan que GEM es necesaria para la correcta reorganización de la cromatina en los loci que controlan la especificación de la identidad celular dependiente de posición. En particular, parece responsable de reclutar factores que modifican la acetilación y metilación de la H3K9 en los promotores de los genes de especificación de identidad celular como mecanismo para el control de su expresión.

GEM es un represor general de la división celular en el meristemo radicular. Reprime las divisiones transversales y, consecuentemente, reduce el tamaño del meristemo. Además, GEM inhibe específicamente el cambio en el plano de división que origina las divisiones longitudinales (anticlinales y periclinales) responsables del incremento en grosor de la raíz. GEM también reduce el potencial de división de células madre y, en conjunto, estos datos confirman que GEM es una proteína crítica para asegurar la correcta formación de patrón y desarrollo de la raíz.

Geminina es una proteína de metazoos que inhibe Cdt1 tras la iniciación de la replicación del DNA y que además controla la expresión génica y la proliferación celular durante la embriogénesis. Nuestra hipótesis es que animales y plantas han desarrollado dos proteínas no relacionadas, geminina y GEM respectivamente, que durante la organogénesis juegan papeles homólogos en la regulación de la transición de células precursoras en estado indiferenciado a células diferenciadas con identidades específicas. Estas proteínas regulan, probablemente en fase G1 del ciclo celular, la expresión de genes involucrados en identidad celular e iniciación de la diferenciación. También interaccionan con Cdt1, un componente de los complejos pre-replicativos involucrado en el licenciamiento de orígenes para la replicación. La interacción de geminina y GEM con Cdt1 y los reguladores transcripcionales es competitiva, lo que sugiere que estas interacciones pueden jugar un papel crítico en la coordinación de la replicación del DNA, la división celular y las decisiones de identidad celular.

Una homología interesante entre GEM y geminina se refiere a su capacidad para interaccionar con Cdt1. Cdt1 es un factor conservado en eucariotas y cuya función está estrictamente controlada para mantener la integridad genómica. En la mayoría de los organismos eucariotas, Cdt1 es una diana clave regulada por diversas vías. Es interesante comentar que plantas con exceso de CDT1 no poseen un fenotipo de re-replicación, sino que sufren un cambio al programa de endociclo. CDT1 es redundantemente enviado a degradación vía proteosoma por complejos que contienen SKP2 o CUL4. El mecanismo de inhibición de CDT1 presente en metazoos, sin embargo, parece no encontrarse presente en plantas, al menos como control de la replicación. Es posible que existan otras estrategias que aún desconocemos, puesto que la eliminación de los mecanismos de degradación no es suficiente para producir un fenotipo de endoreplicación elevada en plantas de Arabidopsis en desarrollo.

Summary

The development of complex multicellular organisms requires the precise specification of diverse cell types at specific times and locations. The coordination of cell fate specification and progression throughout the cell cycle is necessary to achieve a correct developmental progression.

In the *Arabidopsis thaliana* root epidermis, expression of the homeobox *GLABRA2* (*GL2*) gene determines the hair/non-hair cell fate. We have identified a *GL2*-expression modulator, GEM, a novel protein that interacts with the two Cdt1 homologues in Arabidopsis and that is responsible for changes in epidermal cell patterning during root development. GEM interacts with TTG1 (TRANSPARENT TESTA GLABRA1), a WD40-repeat protein involved in *GL2*-dependent cell fate decisions, and modulates both cell division and *GL2* expression. Our studies reveal that GEM is required for a correct chromatin reorganization at loci controlling position-dependent cell fate specification. In particular, it may be responsible for recruiting factors that modify histone H3K9 acetylation and methylation of cell fate specification promoters, as a mechanism to control the expression of patterning genes.

GEM is a general repressor of cell division in the Arabidopsis root meristem. It represses transversal cell division and consequently, reduces meristem size. GEM also specifically inhibits the change in the division plane necessary to generate the longitudinal divisions (anticlinal and periclinal) responsible for the increase in thickness of the root. GEM restricts stem cell division potential too, and altogether, these data confirm that GEM is a critical protein for assuring a proper root patterning and development.

Geminin is a metazoan protein involved in the regulation of DNA replication through its inhibitory activity on Cdt1, but current evidence supports a dual role of geminin as a cellular switch that controls gene expression, DNA replication events and cell proliferation during animal embryogenesis. We suggest animals and plants have evolved two unrelated proteins, geminin and GEM, respectively, that play analogous roles in regulating the transition of precursor cells from an undifferentiated proliferative state to differentiated cells with specific fates during organogenesis. These proteins are involved, probably in early G1 phase of the cell cycle, in regulating the expression of genes involved in cell fate and initiation of differentiation. They also interact with Cdt1, a component of the pre-replication complexes involved in DNA replication licensing in early G1 phase. The interaction of geminin and GEM with Cdt1 and transcriptional regulators is competitive, suggesting that these interactions can play a pivotal role in coordinating DNA replication, cell division and cell fate decisions.

An intriguing analogy between GEM and geminin refers to their ability to interact with Cdt1. In most eukaryotic organisms, Cdt1 is tightly controlled being a key target over which the main control pathways to maintain genome integrity are established. Interestingly, plants with excess of CDT1 do not trigger a re-replication phenotype, but instead they switch to the endocycle program. Arabidopsis CDT1 is redundantly targeted for proteolysis by SKP2- and CUL4-based complexes. The CDT1 inhibition mechanism present in metazoans, however, does not seem to exist in plant cells, at least as a replication control pathway, but other redundant strategies might exist that still remain unknown, since an abrogation of the degradation systems is not enough to produce an endoreplication phenotype in developing Arabidopsis plants.

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Abbreviations used

A	atrichoblast
aa	aminoacid
ACT2	ACTIN 2
APC	anaphase promoting complex
ATP	Adenosine tri-phosphate
bHLH	basic helix loop helix
Brg1	brahma-related gene 1
CAK	CDK-activating kinases
Cdc2	cell division cycle 2
Cdc6	cell division cycle 6
Cdc7	cell division cycle 7
Cdc10	cell division cycle 10
Cdc45	cell division cycle 45
Cdk	cyclin-dependent kinase
Cdt1	Cdc10 dependent transcript 1
ChIP	Chromatin immunoprecipitation
CKI	CDK inhibitory proteins
Col0	Columbia 0 ecotype
CPC	CAPRICE
Cul	cullin
Cy motif	cyclin binding motif
Cyc	CYCLIN
Das	days after sowing
DP	dimerization partner
Ds	double stranded
EDTA	ethylene di-amine tetra-acetic acid
EGL3	ENHANCER OF GLABRA 3
ETC1	ENHANCER OF TRY AND CPC 1
FISH	fluorescence *in situ* hybridization
GA	gibberellin
GEM	*GL2* EXPRESSION MODULATOR
GINS	Go, Ichi, Ni and San complex
GL2	GLABRA 2
GL3	GLABRA 3
Gmn	geminin
GRAM	glucosyltransferase/Rab-like GTPase activators/myotubularins
GST	glutathion S trasferase
GUS	5-bromo-4-chloro-3-indolyl-b-D-glucuronide
HA	haemagglutinin
HBT	HOBBIT
IC	initiation complex
IP	Immunoprecipitation
KDa	kilodalton
KRP	KIP related proteins
Mcm	minichromosome maintenance proteins
MS	Murashige and Skoog Basal Salt Mixture
Nsd	Non-stomatal cell density
ORC	origin recognition complex
PBS	phosphate-buffered saline
PcG	polycomb group
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PIP	PCNA-interaction protein motif

PMSF	phenyl methyl sulphonyl fluoride
PPB	preprophase band
Pre-RC	pre-replication complex
QC	quiescent center
RB	retinoblastoma
RBR	retinoblastoma related
Rbx1	ring-box 1
RPA	replication protein A
RNAi	RNA interference
RT-PCR	reverse transcriptase polymerase chain reaction
s.e.m.	standard error of the mean
SCF	Skp1-Cullin1-F-box protein
SCM	SCRAMBLED
Scmh1	sex comb on midleg homolog 1
SCR	SCARECROW
SD	minimal synthetic defined media
Sd	stomatal density
s.d.	standard deviation
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SHR	SHORTROOT
Si	stomatal index
Skp1	S-phase kinase-associated protein 1
Skp2	S-phase kinase-associated protein 2
Ss	single stranded
STB	STRUBBELIG
SWI–SNF	SWItch/Sucrose Non Fermentable
T	trichoblast
TBE	Tris-borate/EDTA
T-DNA	transferred DNA of the tumor-inducing plasmid of *Agrobacterium tumefaciens*
TE	Tris EDTA buffer
TRN	TORNADO
TrxG	Trithorax Group
TRY	TRYPTICHON
TTG1	TRANSPARENT TESTA GLABRA 1
WB	western blot
WER	WEREWOLF
WOX5	WUSCHEL-RELATED HOMEOBOX 5

Índice

Introducción:
Importancia de la replicación del DNA y el ciclo celular durante la organogénesis

Introducción

1. Ciclo celular

Todos los organismos vivos se componen de células que deben proliferar para asegurar su propia perpetuación. Todos los eventos que tienen lugar en una célula desde una división hasta la siguiente es lo que denominamos el ciclo celular (Fig. 1). Se compone de la coordinación de diferentes procesos, tales como el crecimiento celular, la duplicación del genoma y la distribución entre las dos células hijas, y la división citoplasmática o citoquinesis. Todos estos eventos se dividen operativamente en cuatro etapas conocidas como **fase G1** (crecimiento celular y almacenamiento de nutrientes junto a monitorización de si el ambiente es favorable para la división), **fase S** (síntesis o replicación del DNA), **fase G2** (garantía de que la replicación del DNA se ha completado correctamente) y **fase M o mitosis** (segregación del material genético duplicado y citoquinesis).

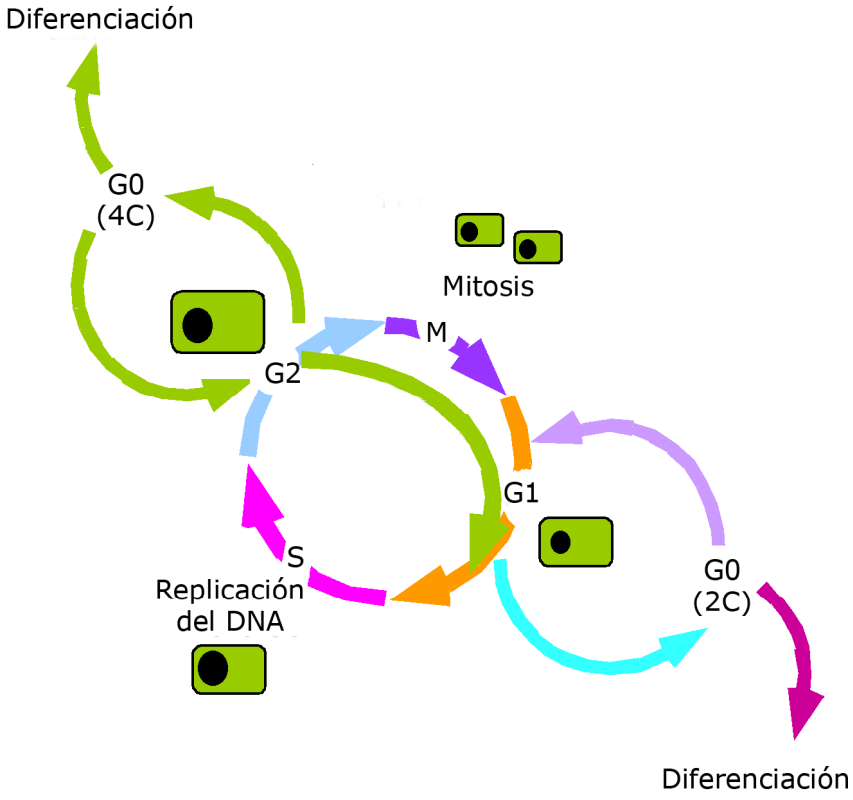

Figura 1: Ciclo celular eucariótico.
Se representa en verde la salida del ciclo celular en G2 y el ciclo de endorreplicación, eventos frecuentes para las células vegetales.

El proceso de organogénesis depende de un balance continuo entre proliferación celular (progresión del ciclo celular) y diferenciación, y en plantas es un proceso único porque incluye tanto el proceso de desarrollo embrionario como el post-embrionario. La planta modelo *Arabidopsis thaliana* ofrece la posibilidad de

combinar aproximaciones bioquímicas, moleculares, celulares y genéticas, lo que la convierten en un potente modelo para el estudio de la integración de la división celular, el crecimiento y el desarrollo en los organismos multicelulares.

Las estrategias básicas del ciclo celular son comunes para todos los eucariotas, aunque existen características específicas del ciclo celular de las plantas que merecen una consideración especial. Las transiciones del ciclo celular en Arabidopsis, como en todos los eucariotas, están controladas por una compleja red que incluye quinasas dependientes de ciclinas (Cdks) como reguladores principales (Gutierrez, 2005; De Veylder et al., 2007). La familia de CDKs de Arabidopsis es una familia compleja de 12 miembros (Vandepoele et al., 2002): las proteínas **CDKA** que contienen la típica secuencia PSTAIRE y son homólogas a Cdc2 de levaduras y son necesarias para las transiciones G1/S y G2/M. Las proteínas **CDKB,** que son CDKs específicas de plantas que se expresan desde la fase S a la M (B1; PPTALRE) y en las fases G2 y M (B2; PPTTLRE). **CDKC** (PITAIRE), que es una CHED quinasa de función desconocida, y **CDKD** y **CDKF,** que funcionan como quinasas activadoras de las CDKs (CAK).

Las ciclinas (Cyc) son las subunidades activadoras de las Cdks. En el genoma de Arabidopsis se han identificado más de 40 ciclinas diferentes (Vandepoele et al., 2002; Wang et al., 2004), muchas más que en cualquier otro eucariota. Es posible que este número elevado de reguladores de ciclo celular se deba a la naturaleza sésil del modo de vida de las plantas. Se cree que el alto número de genes de ciclo celular ayuda al control fino del desarrollo, muy necesario para las plantas ya que éstas no pueden escapar a las condiciones adversas del ambiente, y por lo tanto precisan ajustar su desarrollo a los cambios en las condiciones ambientales.

La actividad Cdk puede ser inhibida mediante la unión de las CKIs (Cdk inhibitory proteins), también conocidas como KRPs (KIP related proteins). En todos los eucariotas los KRPs actúan como reguladores del ciclo celular en respuesta a las señales medioambientales y del desarrollo, mediante la inhibición de la actividad Cdk requerida para la mitosis. Los KRPs también se encuentran involucrados en la regulación de la salida del ciclo celular que precede la diferenciación. En Arabidopsis, los KRPs son esenciales para controlar la actividad CDK durante los endociclos, mostrando una adaptación de sus funciones a las necesidades específicas de las plantas (De Clercq e Inzé, 2006).

Las plantas y los animales también usan la vía E2F/Rb como un mecanismo de

control de la regulación de la transición G1/S. Mientras que Arabidopsis posee múltiples CDKs y KRPs, su genoma contiene sólo un gen relacionado con Rb (*RBR,* RB related) que posee, como sus homólogos de animales, dos bloques de secuencia conservada que forman el dominio llamado "bolsillo A/B", el lugar de anclaje para los factores de transcripción E2F. En células quiescentes (G0) y durante el comienzo de la fase G1, la actividad E2F es reprimida por RBR. Tras la estimulación del crecimiento, RBR es fosforilada por las CDKs y, consecuentemente, pierde su afinidad por los factores E2F. La liberación por RBR conlleva la activación de la expresión de los genes diana de E2F, que lleva irreversiblemente a las células a la fase S (Inzé y De Veylder, 2006; Ramirez-Parra et al., 2007). RBR reprime la actividad E2F no sólo mediante el enmascarado físico del dominio de transactivación de E2F sino que también recluta activamente factores de remodelación de la cromatina, uniendo la vía E2F/Rb al control epigenético (Shen, 2002).

Arabidopsis contiene seis E2Fs (E2Fa, E2Fb, E2Fc, E2Fd/DEL2, E2Fe/DEL1, y E2Ff/DEL3) y dos DPs, con los que heterodimeriza (DPa y DPb). E2Fa y E2Fb actúan como activadores transcripcionales como se demuestra por su capacidad para inducir genes reporteros que se regulan por los elementos activadores E2F consenso. Por el contrario, E2Fc, que no posee un dominio de activación, opera como un regulador negativo (Inzé y De Veylder, 2006; Ramirez-Parra et al., 2007).

La citoquinesis es otro proceso del ciclo celular que difiere bastante entre células vegetales y animales. Las células de las plantas se encuentran rodeadas de una pared celular rígida y, por lo tanto, en vez de formar un anillo contráctil, las células vegetales forman nueva pared entre las dos células hijas (revisado en Jurgens, 2005). El material de la nueva pared se lleva al centro de la célula en vesículas mediante el sistema de microtúbulos formando el fragmoplasto, una organela compleja que consiste tanto en microtúbulos como en filamentos de actina (Backues et al., 2007).

Durante el desarrollo, las células abandonan el ciclo celular para especializarse en una función específica. La salida del ciclo celular mitótico en algunos casos se acompaña de una forma alternativa del ciclo que se conoce como el **ciclo de endorreplicación**. Este ciclo tiene lugar en una gran variedad de tipos celulares de artrópodos y mamíferos, pero es especialmente común en dicotiledóneas (Edgar y Orr-Weaver, 2001). En este ciclo, el material genético duplicado no termina en dos células hijas, sino que se mantiene en la célula madre. La ausencia de citoquinesis

entre rondas de replicación del DNA resulta en la duplicación del contenido de DNA de la célula: 2C, 4C, 8C, 16C… (Kondorosi et al., 2000) (Fig. 1). El papel funcional de la endorreplicación en plantas es aún poco conocido, así como sus mecanismos moleculares, pero se ha relacionado con procesos tales como la diferenciación, la expansión celular, la actividad metabólica y la aptitud para la supervivencia (Sugimoto-Shirasu y Roberts, 2003; Barow, 2006; De Veylder et al., 2007; Caro et al., 2008).

Las células vegetales pueden parar el ciclo celular en G1 en respuesta a condiciones medioambientales adversas, interrumpiendo la proliferación y manteniendo a la célula en un estado metabólicamente activo, llamado **G0** (Muller et al., 1993). En este estado, la célula vegetal mantiene la capacidad para re-entrar en el ciclo en respuesta a estímulos externos, como por ejemplo en la activación de los primordios de raíces adventicias y los brotes axilares o en la formación de los primordios de raíces laterales. La re-entrada en el ciclo celular también ocurre durante la regeneración tras daño. Es posible incluso regenerar plantas completas a partir de células individuales o protoplastos, lo que prueba que las células vegetales mantienen hasta cierto punto el estado de totipotencia (revisado en De Veylder et al., 2007). Los estudios en protoplastos muestran que antes de que las células re-entren en el ciclo celular precisan de un abrupto cambio del estado diferenciado al desdiferenciado. Esta transición se cree basada en un cambio en el estado de la cromatina de la célula, en una descondensación de la cromatina que confiere competencia para el cambio de identidad celular (Zhao et al., 2001).

2. Replicación del DNA

Durante la fase S del ciclo celular, las células tienen que mantener la integridad y ploidía del genoma, asegurando que las dos células hijas reciban la misma cantidad de material genético de la célula madre. Dos procesos son importantes en el control de la replicación: en primer lugar, la formación del complejo pre-replicativo (pre-RC) que licencia el DNA para la replicación, y en segundo lugar, la iniciación de la replicación del DNA.

2.1 El Complejo Pre-replicativo

En procariotas, la replicación comienza en un sitio único y continúa hasta terminar al final del genoma. Si los eucariotas utilizaran este mismo método llevaría varios días duplicar por completo su genoma, ya que poseen unos genomas muchos mayores. Las células eucariotas inician la replicación desde múltiples sitios

dispersos por cada cromosoma y conocidos como **orígenes de replicación**. Los orígenes de replicación eucarióticos dirigen la formación de los pre-RCs que llevan al ensamblaje de las dos horquillas de replicación bidireccionales (DePamphilis, 2006).

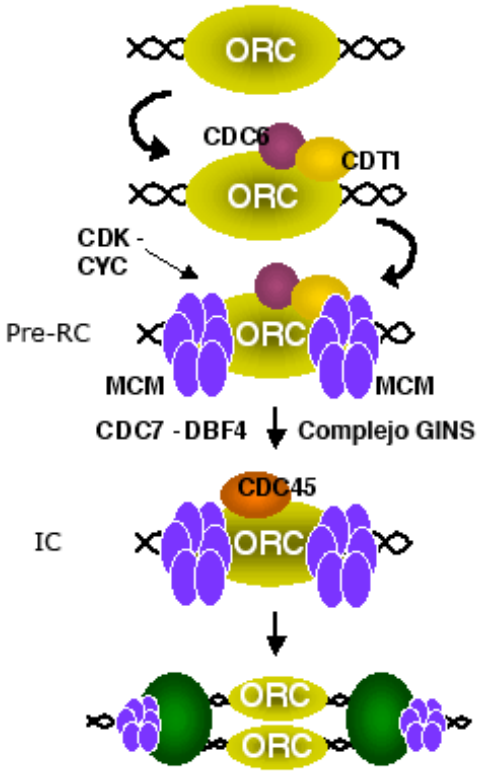

Figura 2: La formación del complejo pre-replicativo (Pre-RC) en la fase G1, su conversión en el complejo de iniciación (IC) en la transición G1/S y el comienzo de la replicación del DNA.

El ensamblaje de los pre-RC comienza durante la fase G1 cuando Cdc6 y Cdt1 son reclutados a los orígenes de replicación donde ORC se encuentra unido. La carga de Cdt1 permite la incorporación del complejo Mcm a la cromatina. La quinasa Cdc7-Dbf4 también es reclutada al origen durante la fase G1 del ciclo celular. Cdc7-Dbf4 y las Cdks promueven el reclutaje de GINS a los orígenes. GINS permite que las Mcms se asocien establemente con otra proteína, Cdc45, que se cree que es un componente esencial de la helicasa Mcm activa. Esta activación de la helicasa resulta en la apertura del DNA del origen y el reclutaje de RPA, la polimerasa α y la primasa a los orígenes para la iniciación de la síntesis de DNA.

El pre-RC se forma en los orígenes de replicación durante la fase G1 para licenciar los orígenes para la replicación. Este licenciamiento conlleva el ensamblaje ordenado de cierto número de factores, incluyendo las 6 subunidades del complejo **ORC** (Complejo de Reconocimiento del Origen), **Cdc6**, **Cdt1** y el complejo **Mcm2-7** (minichromosome maintenance) (Fig. 2). La regulación de la formación del pre-RC es un elemento central en la coordinación de la replicación del DNA dentro del ciclo celular (revisado en Takeda y Dutta, 2005). ORC recluta los factores de iniciación Cdc6 y Cdt1 a los orígenes, que son requeridos para la carga a la cromatina del complejo heterohexamérico Mcm2-7. Cdc6 pertenece a la familia de ATPasas AAA+, mientras que la estructura cristalizada de un fragmento de Cdt1 revela que éste posee un dominio con similitud estructural a un factor bacteriano de la terminación de la replicación que se cree que interacciona físicamente con la helicasa replicativa bacteriana DnaB (Lee et al., 2004). Varias evidencias indican que Mcm2-7 funciona en eucariotas como la helicasa replicativa durante la fase S, viajando con las horquillas de replicación durante la fase S y desenrollando la doble hélice de DNA (Aparicio et al., 1997).

2.2 Cdt1

El factor de licenciamiento **Cdt1** se identificó originalmente como un gen en *S. pombe* que se regulaba por el factor de transcripción Cdc10 (Hofmann y Beach, 1994) (Cdt1, Cdc10 dependent transcript 1) y más tarde se postuló como el factor central en el ensamblaje del pre-RC. Como otros miembros del pre-RC, Cdt1 está conservado en eucariotas, incluyendo *Xenopus laevis* (*Xl*Cdt1) (Maiorano et al., 2000), *D. melanogaster* (Dup/*Dm*Cdt1) (Whittaker et al., 2000), humanos (*Hs*Cdt1) (Nishitani et al., 2001), *S. cerevisiae* (*Sc*Cdt1) (Tanaka y Diffley, 2002) y *Arabidopsis thaliana* (*At*CDT1a y *At*CDT1b) (Castellano et al., 2004).

Mutaciones en *Sp*Cdt1 resultan en un bloqueo de la replicación del DNA y en defectos en el punto de control de la fase S. *Sp*Cdt1 se asocia con el dominio C terminal de *Sp*Cdc6 para, cooperativamente, promover la asociación de las proteínas Mcm a la cromatina (Nishitani et al., 2000). Los mutantes de *S. cerevisiae* parcialmente mermados de Cdt1 replican el DNA a partir de menos orígenes, mientras que las células completamente mermadas de él son incapaces de realizar la carga de Mcm a la cromatina y no pueden iniciar, aunque sí elongar, la síntesis de DNA (Devault et al., 2002).

Mutaciones en Dup (*Dm*Cdt1) causan letalidad embrionaria (Whittaker et al., 2000) y la estabilización de Cdt1 en *C. elegans* causa re-replicación masiva y letalidad en la progenie (Zhong et al., 2003). Debido a estos fenotipos de letalidad, sabemos poco sobre el papel de Cdt1 en organismos completos y sobre si el control del licenciamiento se conecta con los programas de desarrollo y diferenciación. Un trabajo de nuestro laboratorio demostró que niveles alterados de CDT1 o CDC6 poseen efectos específicos de tipo celular en plantas de Arabidopsis: en células de hojas competentes para dividirse, la proliferación celular se ve estimulada, mientras que en células programadas para seguir rondas de endorreplicación asociadas a un programa de diferenciación, se disparan endociclos extra (Castellano et al., 2004). Por lo tanto, CDC6 y CDT1 son dianas centrales en la coordinación de la proliferación celular y la diferenciación durante el desarrollo de la planta.

2.3 Control de la disponibilidad de Cdt1

Es muy importante para las células controlar que los orígenes de replicación sean disparados únicamente una vez cada ciclo, y que ese disparo ocurra sólo durante la fase S, ya que la re-replicación es una fuente de inestabilidad genómica que puede ser desastrosa para la célula. Cdt1 es considerado uno de los componentes

centrales de la regulación del licenciamiento de los orígenes. Se han descrito diversos mecanismos para el control de la disponibilidad de Cdt1 en diferentes organismos, pero aún se desconoce cómo lo controlan las células vegetales. La estrategia de Arabidopsis para el control de Cdt1 se describirá con detalle en el Capítulo 4 de esta Tesis.

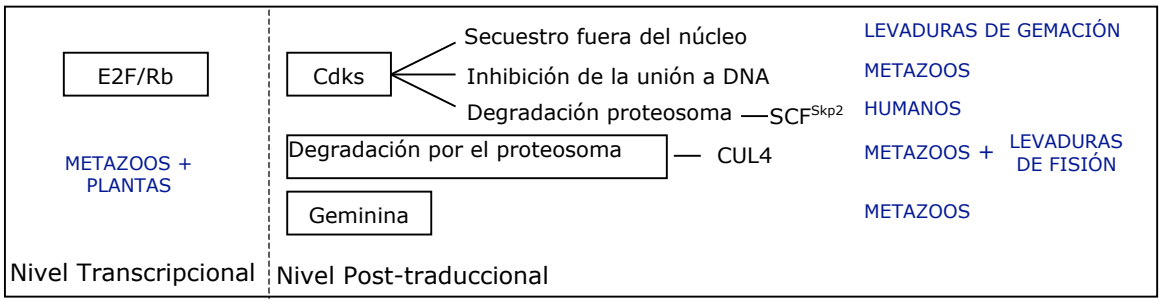

Figura 3: Representación esquemática de los diferentes niveles de control sobre la disponibilidad de Cdt1 para evitar la re-replicación del genoma en diferentes organismos.

2.3.1 Expresión de Cdt1

La vía E2F/Rb es un componente crítico de la regulación del ciclo celular. Las proteínas E2F, como factores de transcripción, regulan positivamente muchos de los genes necesarios para la iniciación de la fase S, y Cdt1 entre ellos tal y como se ha descrito para Drosophila, humanos y plantas (Whittaker et al., 2000; Yoshida y Inoue, 2004; Castellano et al., 2004). Éste es el primer mecanismo mediante el que las células se aseguran de que haya altos niveles de componentes del pre-RC disponibles durante la transición G1/S.

2.3.2 Localización subcelular de Cdt1

La inhibición de cualquier componente del pre-RC durante las fases S, G2 y M debería ser, entonces, suficiente para bloquear la re-replicación; sin embargo, parece que las células usan mecanismos redundantes para inhibir la re-replicación (Fig. 3). Esto es consistente con el descubrimiento de que la alteración individual de la regulación de los componentes del pre-RC no es suficiente para producir re-replicación en células de mamífero (Saha et al., 1998; Herbig et al., 1999; Jiang et al., 1999; Petersen et al., 1999; Delmolino et al., 2001; Nguyen et al., 2001).

La proteína Cdt1 de *S. cerevisiae* se acumula en el núcleo durante la fase G1 y es excluida fuera de él más tarde en el ciclo celular mediante la fosforilación por Cdks (Tanaka y Diffley, 2002). Este mecanismo de secuestro de Cdt1 fuera del núcleo representa un nivel de control post-transcripcional de los componentes del pre-RC, hasta el momento sólo descrito para levaduras de gemación.

2.3.3 Actividad de unión a DNA de Cdt1

En mamíferos se ha encontrado otro nivel de control de los niveles de Cdt1 dependiente de Cdks. Las Cdks fosforilan Cdt1 impidiendo su unión a DNA. En células paradas en fase G2/M, los niveles de Cdt1 no disminuyen, pero la proteína permanece sin unir a la cromatina. Cuando se inactiva Cdk1 en esas células Cdt1 es desfosforilado y vuelve a unirse a la cromatina (Sugimoto et al., 2004), lo que sugiere que las Cdks median la inhibición de la actividad de union a DNA de Cdt1 mediante la fosforilación.

2.3.4 Degradación de Cdt1

El homólogo humano de Cdt1 (*Hs*Cdt1), como su homólogo de *S. pombe* (Hofmann y Beach, 1994), está presente en la célula sólo durante la fase G1 del ciclo celular (Nishitani et al., 2001). Tras el comienzo de la fase S, los niveles de *Hs*Cdt1 disminuyen y apenas si se detecta en células en fase S o G2. A pesar de estas variaciones en los niveles de proteína a lo largo del ciclo celular, los niveles de RNA mensajero se mantienen relativamente constantes. Además, la proteína Cdt1 se acumula en la presencia de inhibidores del proteosoma, lo que sugiere que la degradación dependiente del proteosoma regula los niveles de Cdt1 de humanos durante la progresión del ciclo celular (Nishitani et al., 2001).

Estudios posteriores demostraron que *Hs*Cdt1 podía ser fosforilado por quinasas dependientes de ciclina A (cyclina A/Cdk1 y cyclina A/Cdk2) dependientemente de un motivo de unión a ciclina tipo RXL (motivo Cy) (Liu et al., 2004; Sugimoto et al., 2004). La fosforilación por Cdks resulta en la unión de Cdt1 a la proteína tipo "F-box" Skp2, un componente del complejo ubiquitina ligasa SCF (Skp1-Culina1-proteína F-box), y su posterior degradación por el proteosoma (Li et al., 2003; Liu et al., 2004; Sugimoto et al., 2004; Takeda y Dutta, 2005) (Fig. 4). Por lo tanto, las Cdks regulan Cdt1 mediante proteolisis dependiente de fosforilación. Sin embargo, un mutante de Cdt1 en el motivo Cy, que es refractario a la fosforilación por Cdks y el reconocimiento por SCF^{Skp2}, muestra sólo resistencia parcial a la degradación durante fase S en células Rat-1 (Sugimoto et al., 2004). También en células HeLa se ha observado que los mutantes en Cdt1 deficientes en fosforilación por Cdks y unión a SCF^{Skp2} son aún degradados (Takeda y Dutta, 2005). Estos resultados muestran claramente que existe otro mecanismo que dirige a Cdt1 a la proteolisis durante la fase S.

Recientemente se ha descrito el papel de un complejo ubiquitina ligasa basado en

Cul4 y de PCNA como reguladores de los niveles de Cdt1 durante la fase S (Arias y Walter, 2006; Hu y Xiong, 2006; Nishitani et al., 2006; Senga et al., 2006). El complejo Cul4-DDB1 es un miembro de la familia de complejos ubiquitina ligasa (Petroski y Deshaies, 2005; Dai y Wang, 2006) junto con el complejo SCF basado en Culina 1 (Cardozo y Pagano, 2004; Vodermaier, 2004; Nakayama y Nakayama, 2006). El papel de Cul4 en la regulación de la función de Cdt1 se sugirió por primera vez en *C. elegans*, donde la ablación de Cul4 por RNAi induce re-replicación que es a su vez suprimida por la eliminación de una copia de Cdt1 del genoma (Zhong et al., 2003). El nivel de proteína Cdt1 en *C. elegans* disminuye en las células según éstas entran en la fase S, y esta disminución desaparece con la eliminación de Cul4. Por lo tanto, el complejo ubiquitina ligasa basado en Cul4 regula también la estabilidad de Cdt1, en este caso, durante la fase S del ciclo celular (Fig. 4).

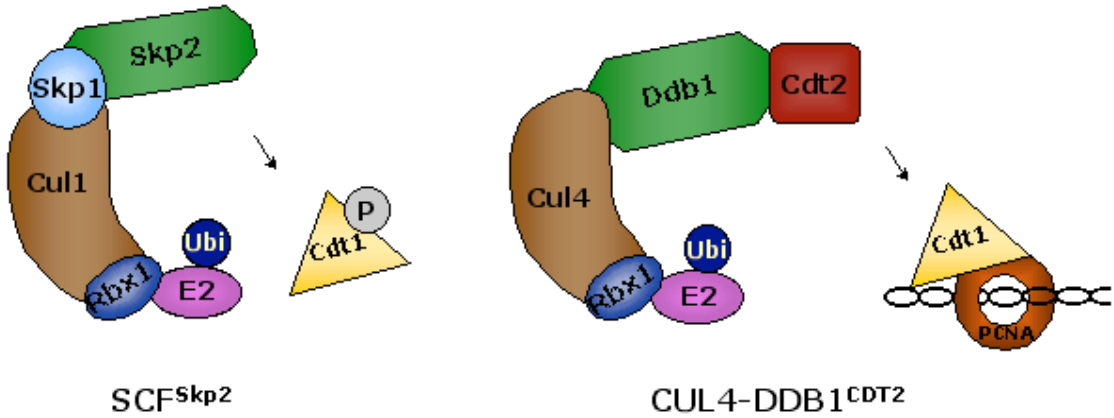

Figura 4: Complejos ubiquitin-ligasa implicados en la degradación de Cdt1 de humanos. A la izquierda, la estructura modular del complejo ubiquitina ligasa SCF^{Skp2}. SCF^{Skp2} dirige a Cdt1 a la degradación tras la fosforilación por Cdk/Ciclinas. A la derecha, el complejo ubiquitina ligasa $CUL4\text{-}DBB1^{CDT2}$. Cul4 dirige a Cdt1 a la degradación tras la unión de éste a PCNA en la cromatina durante la fase S.

Cdt1 posee un motivo de interacción con PCNA en el dominio N terminal de la proteína (caja PIP, PCNA-interacting-protein box: Qxxhxxaa) (Warbrick, 2000; Maga y Hubscher, 2003), que se encuentra conservado de *C. elegans* a mamíferos (Arias y Walter, 2006) (Tabla 1). Tal y como se describió anteriormente, las mutaciones que evitan la unión con Skp2 no pueden estabilizar la proteína Cdt1 de humanos durante la fase S. Es interesante que mutaciones adicionales en el motivo PIP consigan bloquear la degradación de Cdt1 durante la fase S. El silenciamiento de Cul4 o de DDB1 mediante RNAi también estabiliza los mutantes de Cdt1 deficientes en unión a Skp2 (Hu y Xiong, 2006; Nishitani et al., 2006; Senga et al., 2006). Por lo tanto, la regulación proteolítica del Cdt1 de humanos durante la fase S se lleva a cabo mediante dos mecanismos redundantes, la ubiquitinación y degradación mediada por SCF^{Skp2} y Cul4-DDB1.

	Q-x-x-h-x-x-a-a	Posición de los aa
H. sapiens	**Q**-R-R-**V**-T-D-**F**-**F**	3-10
M. musculus	**Q**-S-R-**V**-T-D-**F**-**Y**	3-10
D. melanogaster	**Q**-P-S-**V**-A-A-**F**-**F**	3-10
C. elegans	**Q**-T-A-**V**-T-D-**F**-**F**	14-21
D. rerio	**Q**-A-R-**V**-T-D-**Y**-**F**	3-10
G. gallus	**Q**-L-R-**L**-T-D-**F**-**F**	3-10
X. laevis	**Q**-M-R-**V**-T-D-**F**-**F**	6-13

Tabla 1: Caja de interacción con PCNA (PIP box) en los homólogos de Cdt1.
En la secuencia consenso de la caja PIP, 'h' representa un aminoácido hidrofóbico, 'a' uno aromático y 'x' cualquier aminoácido. Los números a la derecha representan la posición de los aminoácidos dentro de la proteína.

PCNA también se carga a la cromatina durante la reparación del DNA dañado que opera fuera de la fase S. En células humanas, Cdt1 es rápidamente dirigido a degradación por el proteosoma tras daño en el DNA por radiación UV, y esta proteolisis conlleva la ubiquitinación de Cdt1 por la ligasa Cul4-DDB1 (Higa et al., 2003; Hu et al., 2004) y es dependiente de la interacción PCNA-Cdt1 (Hu y Xiong, 2006; Nishitani et al., 2006; Senga et al., 2006). No se conoce muy bien cómo contribuiría la degradación de Cdt1 tras el daño en el DNA a la parada de la entrada en fase S, por lo que el significado biológico de este mecanismo de control de Cdt1 aún no está muy claro (Fujita, 2006).

Arabidopsis conserva varias clases de E3 ubiquitina ligasas que juegan funciones importantes durante el crecimiento cellular y la diferenciación en respuesta al medioambiente (Stone y Callis, 2007). De entre las culinas, Cul2 y Cul5 se encuentran presentes únicamente en metazoos, mientras que Cul1, Cul3 y Cul4 se encuentran conservados en todos los eucariotas (Thomann et al., 2005). El posible papel del complejo SCF basado en CUL1 y/o el de CUL4-DDB1 en el control de los niveles de CDT1 en Arabidopsis no ha sido estudiado aún. En el Capítulo 4 de esta Tesis se expondrán nuestros resultados sobre ello.

2.3.5 Inhibición de Cdt1 por geminina

El descubrimiento de **geminina** en metazoos como inhibidor de la formación de los pre-RCs por interacción con Cdt1 es considerado como su último nivel redundante de regulación. Geminina se descubrió en un escrutinio en busca de proteínas degradadas selectivamente por extractos preparados a partir de huevos de Xenopus en mitosis (McGarry y Kirschner, 1998). Tras este descubrimiento inicial, se caracterizaron homólogos de geminina de Xenopus en la mayoría de los

eucariotas, incluyendo humanos (McGarry y Kirschner, 1998), Drosophila (Quinn et al., 2001), ratón (Yanagi et al., 2002), Medaka (Del Bene et al., 2004) y *C. elegans* (Yanagi et al., 2005), pero hasta el momento no se han descrito homólogos en levaduras ni en plantas.

Geminina es una proteína pequeña de ~33 kDa con características estructurales definidas (revisado en Kroll, 2007):

- un dominio N terminal que incluye una caja de destrucción y una señal de localización nuclear;
- una porción central que incluye una cremallera de leucina atípica con plegamiento de "coiled coil", necesaria para la homodimerización y formación de la super estructura de tetrámero (Okorokov et al., 2004) y para la formación de las dos superficies de interacción con Cdt1 (Benjamin et al., 2004; Lee et al., 2004; Saxena et al., 2004);
- una cola C terminal que no posee dominios reconocibles.

Geminina actúa evitando la carga a la cromatina de las proteínas Mcm sin interferir con la asociación de ORC ni de Cdc6, lo que sugería una función sobre Cdt1 (McGarry y Kirschner, 1998). Esto fue pronto comprobado. Se demostró que geminina humana se asocia con Cdt1 produciendo la inhibición del ensamblaje de los pre-RCs, una inhibición que podía ser revertida mediante la adición de un exceso de *Hs*Cdt1 a los extractos de Xenoupus (Wohlschlegel et al., 2000). Desde entonces, muchos datos se han publicado apoyando el papel de geminina como inhibidor de la función de Cdt1 tras la entrada en fase S (McGarry y Kirschner, 1998; Wohlschlegel et al., 2000; Tada et al., 2001). Durante el progreso de la fase S aparecen niveles altos de geminina, que continúa acumulándose hasta la fase M, cuando es degradada en una reacción dependiente de APC (McGarry y Kirschner, 1998), permitiendo el licenciamiento de los orígenes para el siguiente ciclo.

La interacción geminina/Cdt1 no sólo inhibe la función de Cdt1, sino que también protege a Cdt1 de la degradación por el proteosoma mediante la inhibición de su ubiquitinación (Ballabeni et al., 2004). Geminina está, en este sentido, asegurando niveles basales de Cdt1 durante la fase S y su acumulación durante mitosis. Así pues, la inhibición de la síntesis de geminina durante la fase M lleva al fallo en la formación de los pre-RCs y la replicación del DNA durante el siguiente ciclo celular (Ballabeni et al., 2004).

No se han encontrado ortólogos de geminina ni en organismos unicelulares,

incluyendo levaduras, ni en organismos multicelulares no metazoos, como las plantas, lo que sugiere que es probable que geminina sea específica de al menos algunas formas de organismos multicelulares metazoos (Caro y Gutierrez, 2007). Esta tesis se apoya en diferencias anteriormente observadas en la regulación de la replicación del DNA. Niveles altos de Cdks en G2 y M bloquean la re-replicación del DNA, mientras que la inhibición de la actividad Cdk estimula la re-replicación en levaduras (que no poseen geminina) pero no en metazoos (que contienen geminina como un mecanismo adicional de salvaguarda) (Correa-Bordes y Nurse, 1995; Dahmann et al., 1995; Sun et al., 2000). En la mayoría de estos metazoos, cuando se disminuyen experimentalmente los niveles de Cdk al final de la fase S, las células vuelven a comenzar de nuevo la replicación, sin embargo, esto no lleva a re-replicación, sino que resulta en endorreplicación (Hayles et al., 1994; Moreno y Nurse, 1994; Itzhaki et al., 1997). Este principio se encuentra, curiosamente, conservado también en las células vegetales, donde se requiere una disminución en la actividad Cdk para que las células endorrepliquen (Verkest et al., 2005), aunque las plantas no parecen poseer un homólogo de geminina en sus genomas.

De cualquier modo, asegurar la integridad genómica tras la replicación del DNA no es la única función conocida de geminina. Es tentador pensar que en eucariotas superiores, la presencia de estos mecanismos redundantes para regular la fidelidad de la replicación del DNA ha favorecido que geminina adquiera papeles reguladores adicionales (como se discute en Kroll, 2007). En este contexto, es importante no olvidar que geminina fue identificada concurrentemente en un escrutinio en embriones de *Xenopus laevis* para buscar moléculas que pudieran producir una expansión de la placa neural (Kroll et al., 1998). Ahora sabemos que geminina juega, tanto en los embriones de vertebrados como en los de invertebrados, un papel crítico en la determinación de la identidad neural. Este aspecto será tratado en el Capítulo 2 de esta Tesis, con la discusión de las homologías funcionales entre geminina y una proteína de plantas que interacciona con CDT1 (GEM), identificada en el curso de este trabajo (Caro et al., 2007).

3. El ciclo celular en el contexto de un organismo completo: desarrollo

Cuando se estudia el desarrollo de organismos multicelulares es más apropiado considerar el concepto de proliferación celular que el de ciclo celular o división. La proliferación celular incluye la progresión del ciclo celular, la parada del ciclo y la re-entrada en él, la endorreplicación, la salida a diferenciación y la muerte celular, actuando conjunta y coordinadamente ya que, en organismos multicelulares, la

proliferación celular se ha de coordinar con el crecimiento de los órganos y los programas específicos de desarrollo.

Una hipótesis en auge es que los componentes reguladores del ciclo celular, además de controlar la progresión del mismo, también poseen un importante papel en el control de la proliferación celular en el contexto de un organismo en desarrollo. Alterar el control del ciclo celular posee consecuencias profundas en la organogénesis, aunque las plantas parecen ser muy tolerantes a los cambios en los niveles de reguladores del ciclo celular (Gutierrez, 2005; De Veylder et al., 2007), y en ellas las perturbaciones en la proliferación celular no se encuentran asociadas a muerte celular programada ni a transformaciones oncogénicas, tal y como ocurre en animales. El control de la proliferación celular y la diferenciación durante el desarrollo vegetal depende, también, de la acción concertada de diferentes hormonas. De entre ellas, las auxinas y las citoquininas son las más conocidas por sus consecuencias sobre los reguladores del ciclo celular (Stals e Inzé, 2001; Hartig y Beck, 2006). Además, otras hormonas, como por ejemplo el ácido abscísico, el etileno, el ácido jasmónico y los brasinoesteroides, cuya acción está peor caracterizada, también poseen un probado impacto en la progresión y/o la parada del ciclo celular (del Pozo et al., 2005).

3.1 Balance entre proliferación y diferenciación

Durante el desarrollo, las transiciones entre los programas celulares que regulan la proliferación celular y la diferenciación deben ser coordinadas y controladas temporalmente de manera muy precisa para asegurar que un número apropiado de células formen los correctos patrones de tejidos y órganos. Pero incluso antes de que una célula se diferencie, es absolutamente necesario que adopte la correcta identidad celular. Es por esta razón por la que la coordinación de la proliferación celular y las decisiones de identidad celular se encuentra en la base de cualquier proceso de desarrollo.

3.1.1 Metazoos

El desarrollo depende críticamente del balance entre proliferación y diferenciación. A pesar de que las señales que controlan el balance proliferación-diferenciación *in vivo* siguen siendo desconocidas, hasta el momento se han propuesto algunas moléculas con posible papel en esta transición. El factor regulador del interferón 6 en el desarrollo de la epidermis (Richardson et al., 2006), el receptor α de estrógeno en la diferenciación de progenitores neurales (Ciana et al., 2003), Sonic hedgehog durante la organogénesis del pulmón de ratón (Li et al., 2004) y N-myc

durante la diferenciación neural (Ciani et al., 2004) son algunos ejemplos de moléculas involucradas en este cambio.

Aparte de su papel en la regulación de la replicación del DNA, **geminina** posee una función adicional recientemente descubierta. Es un conocido coordinador de la proliferación, identidad y diferenciación celular. En casi todos los contextos celulares estudiados hasta la fecha, la expresión de geminina correlaciona fuertemente con los estados celulares progenitores activamente en división, en el embrión y en el adulto, mientras que se muestra reducida antes o coincidentemente con la parada del ciclo celular (Quinn et al., 2001; Wohlschlegel et al., 2002; Xouri et al., 2004; Montanari et al., 2005). Además, geminina correlaciona con células en proliferación en muchos cánceres, marcando neoplasmas agresivos ya que caracteriza específicamente células con una elevada tasa de progresión del ciclo celular y disminuida fase G1 (Wohlschlegel et al., 2002; Gonzalez et al., 2004).

Muchos estudios han probado la necesidad de geminina para la regulación del desarrollo embrionario de **vertebrados**. En *Xenopus laevis*, geminina regula la identidad neural durante la gastrulación mediante su dominio N terminal (Kroll et al., 1998; McGarry, 2002). Se ha mostrado que el dominio C terminal de geminina regula la neurogénesis tardía, cuando los precursores neurales abandonan el ciclo celular y se diferencian mediante la antagonización de la actividad de Brg1 (una subunidad catalítica del complejo regulador de la cromatina SWI/SNF) (Seo et al., 2005).

También se ha postulado un papel para geminina en el control de la expresión génica y la actividad de Hox. Los genes *Hox* de vertebrados se agrupan en cuatro grupos y se expresan con patrones solapantes a lo largo del eje antero-posterior del embrión. Tras el establecimiento de los patrones de expresión de los genes *Hox*, éstos se mantienen durante las siguientes divisiones a través de los grupos Trithorax y Polycomb (Gebuhr et al., 2000; Ringrose y Paro, 2004). Geminina puede asociarse directamente con las proteínas Hox y con la proteína Scmh1 del grupo Polycomb durante la embriogénesis del pollo (Luo et al., 2004), impactando negativamente en la función y la expresión de los genes Hox para regular el patrón axial antero-posterior (Luo et al., 2004; Saxena et al., 2004). Geminina puede también bloquear la expresión de los genes homeobox en la retinogénesis de Medaka, donde se une y antagoniza la función de la proteína homeodominio Six3 (Del Bene et al., 2004).

Otro papel potencial de geminina en el desarrollo lo podemos ver en el ojo del **invertebrado** Drosophila (Quinn et al., 2001), que se asemeja a la actividad de los homólogos de vertebrados de geminina. Los experimentos de ganancia y pérdida de función de *Dm*geminina en el sistema nervioso apoyan su función de regulación de la identidad neuronal y la diferenciación (Quinn et al., 2001).

En conjunto, estos datos definen características comunes de la actividad de geminina en varios contextos de desarrollo como un coordinador del ciclo celular y de las vías de regulación transcripcional que controlan la toma de decisiones de identidad celular y la diferenciación durante la organogénesis (revisado en Kroll, 2007). En el Capítulo 2 de esta Tesis se presentará una evaluación comparativa del papel jugado por geminina en animales y GEM en plantas, dos proteínas aparentemente no relacionadas que, en cualquier caso, presentan una serie de interesantes homologías mediante su participación en el control de la división celular y los cambios en las marcas epigenéticas de las histonas en loci específicos durante el desarrollo.

3.1.2 Plantas

Para llegar a entender el proceso del desarrollo es útil estudiar sistemas simples. Arabidopsis es ampliamente utilizada como planta modelo por genéticos moleculares para el estudio tanto de problemas básicos como aplicados. El desarrollo en plantas y animales, a pesar de compartir las estrategias fundamentales, muestra importantes diferencias. Al contrario que los animales, las plantas se desarrollan continuamente y en respuesta al ambiente. Esta plasticidad en el desarrollo reside, al menos parcialmente, en que los órganos de las plantas se producen constantemente a partir de grupos de células madre indiferenciadas que se encuentran en los meristemos. En Arabidopsis se han usado diferentes modelos para estudiar la proliferación celular dentro del contexto de desarrollo, principalmente el desarrollo embrionario (Willemsen y Scheres, 2004), el meristemo apical aéreo (Carles y Fletcher, 2003), el meristemo floral (Lohmann y Weigel, 2002) y el meristemo radicular (Benfey y Scheres, 2000). Debido a la simplicidad de su organización, hemos elegido la raíz de Arabidopsis como modelo para el estudio de la coordinación entre proliferación y diferenciación durante el desarrollo.

Las raíces de Arabidopsis se pueden considerar un grupo de cilindros concéntricos (revisado en Benfey y Scheres, 2000). Las cuatro capas exteriores, la epidermis, el cortex, la endodermis y el periciclo, rodean el tejido vascular situado en la zona más interna de la raíz. La epidermis se encuentra compuesta por dos tipos

celulares, las células que forman pelos radiculares y las que no. El cortex y la endodermis se componen por un único tipo celular y suelen incluir ocho células cada capa. El periciclo está formado por células que pueden iniciar la formación de raíces laterales, y en el centro de la raíz podemos encontrar el tejido vascular (Fig. 5).

Dentro de la raíz en crecimiento, las nuevas células se originan en el ápice distal, en el meristemo. Aquí encontramos grupos de células iniciales (equivalentes a las células madre de animales) que se encuentran situadas alrededor de un grupo de células de mermada capacidad de división y que forman el centro quiescente (QC).

El QC es la fuente de producción de la señal que mantiene a las células madre en su estado. Cada grupo de células iniciales seguirá un patrón de división estereotipado para generar su progenie. Debido a que las células vegetales no se pueden mover las unas respecto a las otras, las divisiones de las células iniciales dan lugar a columnas o filas de células. La relación entre células de una fila refleja su edad, de forma que las células más jóvenes se encuentran más cerca del ápice de la raíz. Por lo tanto, todos las etapas del desarrollo se encuentran presentes en cada raíz, y la anatomía de ésta refleja su ontogenia (Benfey y Scheres, 2000).

En las células meristemáticas debe conseguirse un balance entre producción celular y diferenciación mediante el control de su tasa de división. Si este control se altera, el meristemo podría no mantenerse o agrandarse anormalmente. La relación entre la especificación de la identidad celular y la formación de patrón mediante divisiones celulares en la raíz, además de la función de GEM sobre ambas, se discutirá en detalle en el Capítulo 3 de esta Tesis.

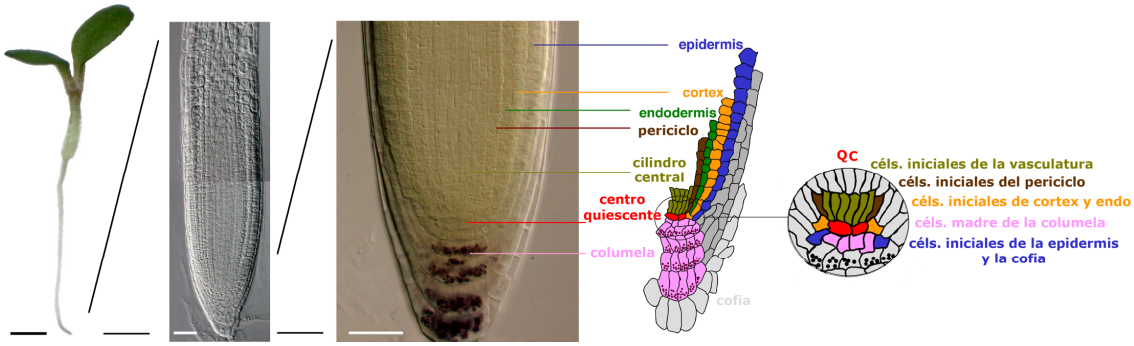

Figura 5: Imagen y representación esquemática de un meristemo radicular de Arabidopsis. De izquierda a derecha, una plántula de Arabidopsis (barra, 0.5 mm), una raíz montada con cloralhidrato (barra, 40 μm) y una raíz teñida con lugol, donde los gránulos de almidón de las células de la columela aparecen de color morado (barra, 40 μm). A la derecha, esquema donde se representan los diferentes tipos celulares y su organización en el meristemo de la raíz y un detalle del centro quiescente (QC) y las células iniciales que lo rodean.

Debido a la accesibilidad de la epidermis y a que ésta consiste en tan sólo dos tipos celulares, se ha convertido en un modelo muy utilizado para el estudio de la diferenciación y la formación de patrón en plantas. La distribución de los tipos celulares "pelo" y "no pelo" en la epidermis varía en las diferentes especies vegetales (revisado en Schiefelbein et al., 1997). En algunas, no existe un patrón aparente; en otras, incluyendo muchas monocotiledóneas, la identidad celular se encuentra asociada a una división asimétrica donde la célula más pequeña se diferencia a pelo radicular mientras que la célula mayor genera una o más células maduras sin pelo. En un tercer grupo de plantas, que incluye Arabidopsis y otros miembros de las Brasicáceas, se genera un patrón dependiente de la posición de las células epidérmicas. En este caso, las células que se encuentran entre dos células de la capa subyacente, el cortex, se especifican como tricoblastos (T), mientras que las células epidérmicas que se sitúan sobre una única célula cortical se especifican como atricoblastos (A) y no desarrollarán pelo.

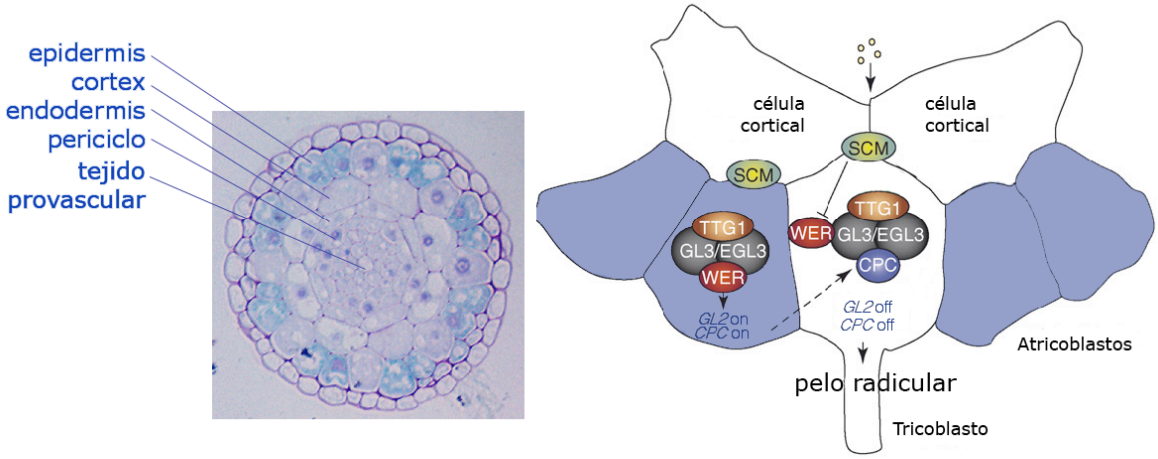

Figura 6: Sección transversal del meristemo de una raíz de Arabidopsis (izquierda) y esquema de la red transcripcional que controla la expresión de *GL2* y *CPC* en la epidermis del meristemo radicular de Arabidopsis para determinar la identidad celular de tricoblasto o atricoblasto (derecha).

Las células tipo "no pelo" se originan en las filas celulares localizadas en la posición "A" y requieren la actividad de la proteína homeodominio GLABRA2 (GL2) (Rerie et al., 1994; Masucci et al., 1996), la proteína de repeticiones WD40 TRANSPARENT TESTA GLABRA 1 (TTG1) (Galway et al., 1994; Larkin et al., 1999), la proteína de dos repeticiones MYB R2R3 WEREWOLF (WER) (Lee y Schiefelbein, 1999), y dos proteínas básicas de la familia hélice-bucle-hélice (bHLH) GLABRA3 (GL3) y ENHANCER OF GLABRA3 (EGL3) (Bernhardt et al., 2003). Las proteínas GL3 y EGL3 interaccionan físicamente con WER y TTG1, y cada una de ellas es necesaria para la transcripción de *GL2* en las células en posición "A", lo que implica que el complejo transcripcional WER-GL3/EGL3-TTG1 regula posicionalmente el destino cellular de "no-pelo" (Hung y Hultgren, 1998; Lee y Schiefelbein, 1999; Payne et al., 2000;

Bernhardt et al., 2003) (Fig. 6).

Las células tipo "pelo" se especifican en filas epidérmicas situadas fuera de la union longitudinal entre células corticales (la posición "T") mediante la acción de 3 proteínas de una sóla repetición R3 MYB, CAPRICE (CPC), TRIPTYCHON (TRY), y ENHANCER OF TRY y CPC1 (ETC1) (Wada et al., 1997; Schellmann et al., 2002; Kirik et al., 2004). Se ha propuesto que las proteínas CPC, TRY, y ETC1 promueven el destino de "pelo" mediante el desplazamiento de WER del complejo WER-GL3/EGL3-TTG1 en la posición T (Dolan y Costa, 2001; Schellmann et al., 2007). La proteína CPC es capaz de moverse de las células A a las T (Wada et al., 2002; Kurata et al., 2005), lo que implica que CPC, y quizá otras MYB de una sóla repetición, actúa mediante un mecanismo de inhibición lateral para especificar el patrón de pelos radiculares (Lee y Schiefelbein, 2002; Schellmann et al., 2007) (Fig. 6). Recientemente, una quinasa codificada por el gen *SCRAMBLED* (*SCM*) (Fig. 6), ha sido involucrada como mediadora de la señal posicional y de la expresión de los genes *CPC*, *GL2*, y *WER* (Kwak et al., 2005). Sin embargo, aún se desconoce si SCM interacciona con los genes involucrados en patrón ya conocidos o el mecanismo empleado.

Xu et al., (2005) mostraron que la acetilación de las histonas posee un papel importante en la regulación de la expresión dependiente de posición de los genes de patrón en la epidermis de raíz de Arabidopsis. Más tarde, Costa y Shaw, (2006) mostraron que se requieren estados alternativos de organización de la cromatina alrededor del locus de *GL2* para controlar la identidad celular de forma dependiente de posición y que el estado de la cromatina alrededor del locus *GL2* se reorganiza cada ciclo celular, lo que determina que *GL2* se transcriba o no en las células hijas.

En el Capítulo 1 de esta Tesis se abordará el estudio del papel de una nueva proteína identificada durante el curso de este trabajo (GEM) en el control del potencial de división de las células epidérmicas del meristemo radicular y las modificaciones de las histonas de los promotores de los genes de formación de patrón *GL2* y *CPC*.

Esfuerzos futuros deberán dirigirse a la identificación de las bases moleculares que operan en la coordinación entre división celular, decisiones de identidad y diferenciación celular. Este tipo de análisis muestran cómo el estudio comparativo de procesos básicos en modelos animales y vegetales son muy útiles para profundizar en el conocimiento de la organogénesis en organismos multicelulares.

Referencias

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Introduction:
Relevance of DNA replication and cell cycle control during organogenesis

Introduction

1. Cell cycle

All living organisms are composed of cells that must proliferate to assure their own self-perpetuation. The whole of the events that take place in a cell from one division to the next one is what we know as the **cell cycle** (Fig. 1). It comprises different processes, such as cellular growth, genome duplication and distribution between the two daughter cells and cytoplasmic division or cytokinesis. All these events are operationally divided into four different stages known as **G1 phase** (cell growth and nutrients storage plus monitoring if the environment is favourable for division), **S phase** (DNA replication or synthesis), **G2 phase** (assurance that DNA replication has been completed successfully) and **M phase or mitosis** (segregation of the duplicated genetic material and cytokinesis).

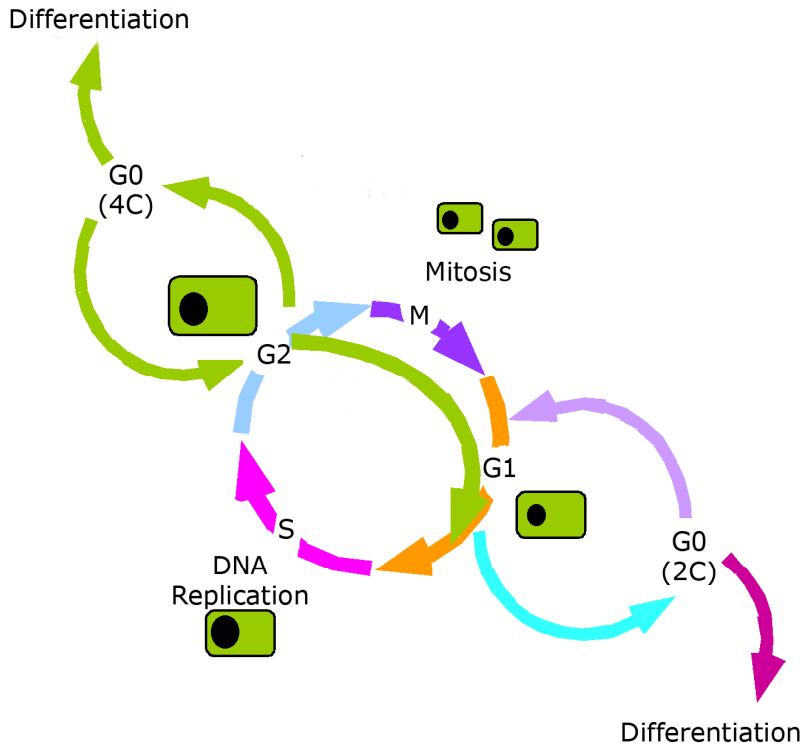

Figure 1: Eukaryotic cell cycle.
We have represented in green cell cycle exit in G2 and the endoreplicative cycle, frequent events for plant cells.

In multicellular organisms, organogenesis depends on a continuous balance between cell proliferation and differentiation, but in plants it is a unique process because it takes place during embryogenesis and also during post-embryonic development. This special feature, together with the possibility to combine biochemical, molecular, cellular, genetic and functional genomic approaches, makes

the model plant *Arabidopsis thaliana* a very powerful model to study the integration of cell division, growth, and development of multicellular organisms.

The basic strategies of the cell cycle are common to all eukaryotes, though there are some specific features of the plant cell cycle that deserve a special consideration. Cell cycle transitions in Arabidopsis, like in all eukaryotes, are controlled by a complex network that involves cyclin-dependent kinases (Cdks) as key regulators (for a review see Gutierrez, 2005; De Veylder et al., 2007). Nevertheless, the Arabidopsis CDK family is very complex and comprises 12 members (Vandepoele et al., 2002). **CDKA** is the typical PSTAIRE-containing CDK, homologous to yeast Cdc2 and necessary for G1/S and G2/M transitions. **CDKB** proteins are plant-specific CDKs that are expressed from S to M phase (B1; PPTALRE) and in G2 and M phases (B2; PPTTLRE). **CDKC** (PITAIRE) is a CHED-related kinase with no known cell cycle function and **CDKD** and **CDKF** function as CDK-activating kinases (CAK).

Cyclins (Cyc) are the Cdk activator subunits. More than 40 different cyclins have been identified in the Arabidopsis genome (Vandepoele et al., 2002; Wang et al., 2004), many more than in any other eukaryote. The high number of cell cycle genes is thought to help the fine control of development, it is possible that this is due to the sessile nature of plant lifestyle; since they cannot escape adverse conditions they need to adjust their development to the changes in the environment.

Cdk activity can be inhibited by binding of CKIs (CDK inhibitory proteins), also known as KRPs (KIP related proteins). In all eukaryotes KRPs act as regulators of the cell cycle in response to environmental and developmental cues by inhibiting Cdk activity and thus producing a mitosis arrest. KRPs are also involved in regulating cell cycle exit before differentiation. In Arabidopsis, KRPs are essential to control Cdk activity in endocycling cells, showing an adaptation of their functions towards the specific needs of plants (De Clercq and Inzé, 2006).

Plants and animals use the E2F/Rb pathway as a major mechanism to control cell division and regulate G1/S transition. Whereas Arabidopsis encodes multiple CDKs, cyclins, and KRPs, its genome contains only one Rb-related gene (*RBR,* RB related) that has, like its animal counterparts, two blocks of conserved sequences that form the so-called A/B pocket domain, the place where E2F transcription factors bind. In growth arrested cells (G0) and during early G1 phase, the E2F activity is repressed

by RBR binding. Upon growth stimulation, RBR is phosphorylated by CDKs and consequently looses its affinity for E2F. This release allows the activation of E2F-target genes, which irreversibly commits cells to DNA replication (S phase) (Inzé and De Veylder, 2006; Ramirez-Parra et al., 2007). RBR has been shown to repress E2F activity not only by physically masking the E2F transactivation domain, but also by actively recruiting chromatin-remodeling factors to target genes promoters, linking the E2F/Rb pathway to epigenetic control (Shen, 2002).

Arabidopsis contains six E2Fs (E2Fa, E2Fb, E2Fc, E2Fd/DEL2, E2Fe/DEL1, and E2Ff/DEL3) and two DPs, the heterodimeric parterns of E2F (DPa and DPb). E2Fa and E2Fb act as transcriptional activators as illustrated by their ability to induce reporter genes harboring the E2F consensus cis-activating elements. By contrast, E2Fc, which lacks a strong activation domain, operates as a negative regulator of the E2F-responsive genes (Inzé and De Veylder, 2006; Ramirez-Parra et al., 2007).

Plant cytokinesis is another feature of plant cell cycle that differs significantly from that of animal cells. Plant cells are surrounded by a rigid cell wall, and thus, instead of forming a contractile ring, plant cells divide by the formation of new walls between the two daughter cells (reviewed in Jurgens, 2005). New cell wall material is brought to the centre of the mother cell in vesicles by the microtubule system forming the phragmoplast, a complex organelle consisting of both microtubules and actin filaments (Backues et al., 2007).

During development, cells exit cell cycle and start to differentiate to become specialized in a specific function. Exit from the mitotic cell cycle is in some cases accompanied by an alternative form of the cell cycle, that is the **endoreplication cycle**. This cycle occurs in a wide variety of cell types in arthropods and mammals, but is especially common in dicots (Edgar and Orr-Weaver, 2001). In this cycle, the duplicated genetic material does not end up in two daughter cells, but instead, remains in the mother cell. The absence of cytokinesis between successive rounds of DNA replication results in the duplication of the DNA content of the cell: 2C, 4C, 8C, 16C… (Kondorosi et al., 2000) (Fig. 1). The functional role of endoreplication in plants and the molecular mechanisms involved in it are still today poorly understood, but have been related to processes such as cell differentiation, cell expansion, metabolic activity and fitness for survival (Sugimoto-Shirasu and Roberts, 2003; Barow, 2006; De Veylder et al., 2007; Caro et al., 2008).

Cells can arrest cell cycle in G1 in response to adverse environmental conditions,

interrupting proliferation and keeping the cell in a metabolic active state called **G0** (Muller et al., 1993). In this state, plant cells retain the ability to re-enter the cell cycle in response to external stimuli, e.g. the activation of adventitious root primordia and dormant axilary buds or the start of lateral root primordia. Cell cycle re-entry also occurs during organ regeneration after injury. Furthermore, even complete new plants have been regenerated from single cells or protoplasts, which proves that plant cells retain to some point the status of totipotency (reviewed in De Veylder et al., 2007). These studies on protoplasts have shown that before cells re-enter the cell cycle, they need to undergo an abrupt switch from the differentiated to the dedifferentiated status. This transition is supposed to be based on a change in the cell's chromatin status, a chromatin decondensation that will confer competence for the cell fate switch (Zhao et al., 2001).

2. DNA replication

During the S phase of the cell cycle, cells have to duplicate their genetic material maintaining the integrity and ploidy of the genome, assuring that daughter cells receive the same content of genetic material from the mother cell. Two steps are important during the control of the replication cycle: first, the formation of pre-replicative complexes (pre-RCs) that license DNA for replication, and second, the initiation of DNA replication.

2.1 The Pre-replicative Complex

In prokaryotes, replication begins from a single site and continues until it terminates at the end of the genome. If eukaryotes used the same strategy, it would take several days to completely duplicate their genome, since they bear a significantly larger genome. To solve this problem, eukaryotic cells initiate replication from multiple sites scattered throughout each chromosome and known as **replication origins**. Eukaryotic origins of replication direct the formation of the pre-RCs leading to the assembly of two bidirectional DNA replication forks (DePamphilis, 2006).

The pre-RCs form at origins of replication during the G1 phase to license origins for replication. The licensing step involves the ordered assembly of a number of replication factors including **ORC** 6 subunits (the Origin Recognition Complex), **Cdc6**, **Cdt1**, and the **Mcm2–7** (minichromosome maintenance) complex (Fig. 2). The regulation of pre-RCs formation is a key element of the coordination of DNA replication within the cell cycle (reviewed in Takeda and Dutta, 2005).

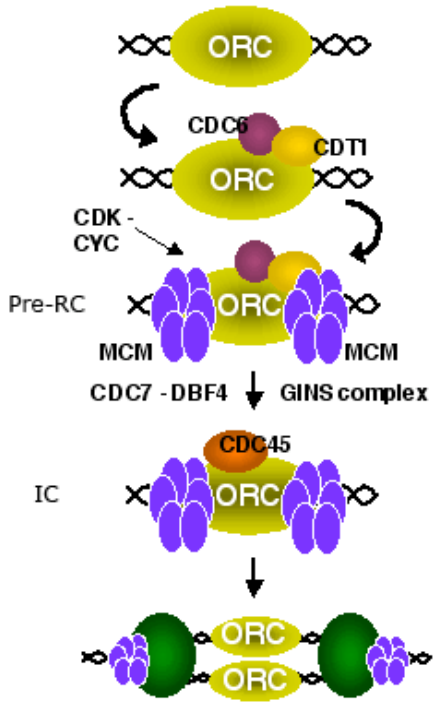

Figure 2: The formation of the pre-RC in G1 phase, its conversion in the initiation complex (IC) at the G1–S phase transition, and the beginning of DNA replication.

Assembly of the pre-RC begins during the G1 phase when Cdc6 and Cdt1 are recruited to replication origins where ORC is bound. Cdt1 loading enables Mcm recruitment to chromatin. The Cdc7-Dbf4 kinase is also recruited to the origin during G1 phase. Cdc7-Dbf4 and Cdk, promote the recruitment of GINS to origins. GINS then allows Mcm to associate stably with another protein, Cdc45, which is thought to be an essential component of the active Mcm helicase. The activation of the helicase allows the initiation of the melting of DNA and recruits RPA, DNA polymerase α and primase to the origins for the initiation of DNA synthesis

ORC recruits the initiation factors Cdc6 and Cdt1 to origins, which are both required for the loading of the heterohexameric Mcm complex. Cdc6 belongs to the AAA+ family of ATPases, while the crystal structure of a fragment of Cdt1 reveals that it has a domain bearing structural resemblance to a bacterial replication terminator protein that is believed to physically interact with the bacterial replicative helicase DnaB (Lee et al., 2004). Several lines of evidence indicate that Mcm functions as the replicative helicase during S phase, travelling with the replication fork during S phase and unwinding the double helix of DNA (Aparicio et al., 1997).

2.2 Cdt1

The licensing factor **Cdt1** was originally identified as a gene in *S. pombe* whose expression was regulated by the Cdc10 transcription factor (Hofmann and Beach, 1994) (Cdt1, Cdc10 dependent transcript 1) and has later been postulated as a key factor in pre-RCs assembly. Like other members of the pre-RC complex, Cdt1 is conserved in eukaryotes including *Xenopus laevis* (*Xl*Cdt1) (Maiorano et al., 2000), *D. melanogaster* (Dup/*Dm*Cdt1) (Whittaker et al., 2000), humans (*Hs*Cdt1) (Nishitani et al., 2001), *S. cerevisiae* (*Sc*Cdt1) (Tanaka and Diffley, 2002) and *Arabidopsis thaliana* (*At*CDT1a y *At*CDT1b) (Castellano et al., 2004).

Mutations in *Sp*Cdt1 result in a block of DNA replication and in defects in the S-phase checkpoint. *Sp*Cdt1 has been shown to associate with the C terminus of *Sp*Cdc6 to cooperatively promote the association of Mcm proteins with chromatin

(Nishitani et al., 2000). *S. cerevisiae* mutants partially depleted of Cdt1 replicate DNA from fewer origins, whereas fully depleted cells fail to load Mcm on chromatin and fail to initiate but not to elongate DNA synthesis (Devault et al., 2002).

Strong mutations in Dup (*Dm*Cdt1) cause embryonic lethality (Whittaker et al., 2000) and *C. elegans* Cdt1 stabilization causes massive DNA re-replication and lethality among the progeny (Zhong et al., 2003). These lethal phenotypes explain why information on the role of Cdt1 in whole organisms is very limited, and support the idea that licensing control interfaces with differentiation and developmental programs. A work from our laboratory showed that altered CDT1 or CDC6 levels have cell type-specific effects in developing Arabidopsis plants: in leaf cells competent to divide, cell proliferation is stimulated, whereas in cells programmed to undergo differentiation-associated endoreplication rounds, extra endocycles are triggered (Castellano et al., 2004). Thus, CDC6 and CDT1 are key targets for the coordination of cell proliferation and differentiation during plant development.

2.3 Cdt1 availability control

It is very important for cells to control that origins are only fired once per cell cycle, and that firing happens only during S phase, since re-replication is a source of genomic instability that can be disastrous for the cell. Cdt1 is considered a key component in the regulation of origin licensing. Several mechanisms for Cdt1 availability control have been described for different organisms, but knowledge about plants is still lacking. Our studies to define the Arabidopsis strategy on CDT1 control will be described in detail in Chapter 4 of this Thesis.

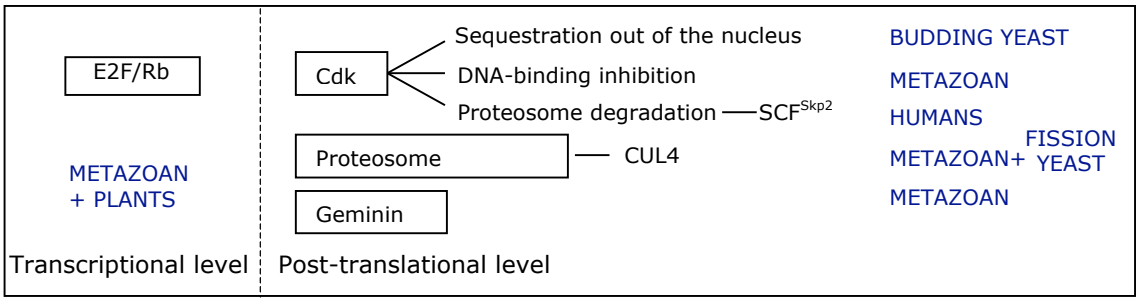

Figure 3: Schematic representation of the different levels of control over Cdt1 availability to avoid genome re-replication in different organisms.

2.3.1 Cdt1 expression

The E2F/Rb pathway is a critical component of cell cycle regulation. E2F proteins, as transcription factors, positively regulate many of the genes required for initiation

of S phase, and Cdt1 among them, as described for Drosophila, humans and plants (Whittaker et al., 2000; Yoshida and Inoue, 2004; Castellano et al., 2004). This is the first way in which cells assure that high levels of pre-RC components are available at G1/S transition.

2.3.2 Cdt1 subcellular localization

Inhibition of any component of the pre-RC during S, G2, and M phases should be, in theory, sufficient to block re-replication; however, it seems that cells use redundant mechanisms to inhibit re-replication (Fig. 3). This is consistent with the finding that alteration of the regulation of individual pre-RC components fail to induce re-replication in mammalian cells (Saha et al., 1998; Herbig et al., 1999; Jiang et al., 1999; Petersen et al., 1999; Delmolino et al., 2001; Nguyen et al., 2001).

S. cerevisiae Cdt1 protein accumulates in the nucleus during G1 phase and is excluded from it later in the cell cycle by Cdk phosphorylation (Tanaka and Diffley, 2002). This mechanism of Cdt1 sequestration outside the nucleus represents a post-translational level of pre-RC components control, at present only described for budding yeast.

2.3.3 Cdt1 DNA binding activity

In mammals, another Cdk-dependent level of control has been described, consisting on the ability of Cdks to phosphorylate Cdt1 impairing its DNA binding activity. In cells arrested around G2/M phase, levels of Cdt1 are not lowered, but the protein remains detached from chromatin. When Cdk1 is inactivated in such cells, Cdt1 is dephosphorylated and rebinds to chromatin (Sugimoto et al., 2004), suggesting that Cdks mediate the inhibition of Cdt1 DNA binding activity by phosphorylation.

2.3.4 Cdt1 degradation

Human Cdt1 (*Hs*Cdt1), as its *S. pombe* homologue (Hofmann and Beach, 1994), is present only during G1 phase of the cell cycle (Nishitani et al., 2001). After S-phase onset, *Hs*Cdt1 levels decrease, and it is hardly detected in cells in early S-phase or G2. Despite these variations in Cdt1 protein levels during the cell cycle, the mRNA level of Cdt1 remains relatively constant at the different cell cycle stages. Cdt1 protein accumulates in the presence of proteasome inhibitors

suggesting that proteasome-dependent degradation is regulating human Cdt1 levels during the cell cycle (Nishitani et al., 2001).

Further studies showed that human Cdt1 can be phosphorylated by cyclin A-dependent kinases (cyclin A/Cdk1 and cyclin A/Cdk2) depending on its RXL-type cyclin-binding motif (Cy motif) (Liu et al., 2004; Sugimoto et al., 2004). Cdk phosphorylation results in Cdt1 binding to the F-box protein Skp2, a component of the SCF (Skp1-Cullin1-F-box protein) ubiquitin ligase complex, and its subsequent degradation (Li et al., 2003; Liu et al., 2004; Sugimoto et al., 2004; Takeda and Dutta, 2005) (Fig. 4). Thus, in human cells Cdks regulate Cdt1 via phosphorylation-dependent proteolysis. However, a Cdt1 Cy mutant, which is refractory to Cdk phosphorylation and SCF^{Skp2} recognition, showed only partial resistance to degradation during S phase in Rat-1 cells (Sugimoto et al., 2004). In HeLa cells, it has also been observed that Cdt1 mutants deficient in Cdk phosphorylation, and subsequent SCF^{Skp2} binding, are still degraded (Takeda and Dutta, 2005). These findings clearly show that, apart from the SCF-mediated mechanism, there exists a separate mechanism that also targets Cdt1 for proteolysis during S phase. Recently, Cullin 4 (Cul4)-based ubiquitin ligase complex and PCNA have been involved in a new proteolytic pathway regulating Cdt1 levels during S phase (Arias and Walter, 2006; Hu and Xiong, 2006; Nishitani et al., 2006; Senga et al., 2006). The Cul4-DDB1 complex is a member of the diverse ubiquitin ligase family (Petroski and Deshaies, 2005; Dai and Wang, 2006), together with the SCF complex based on Cul1 (Cardozo and Pagano, 2004; Vodermaier, 2004; Nakayama and Nakayama, 2006). Involvement of Cul4 in the regulation of Cdt1 function was first suggested in *C. elegans*, in which ablation of Cul4 by RNAi induces re-replication, which in turn is suppressed by removal of one genome copy of Cdt1 (Zhong et al., 2003). The level of *C. elegans* Cdt1 protein decreases as cells enter S phase, and this decrease disappears with Cul4 depletion. Thus, Cul4 ubiquitin ligase appears to regulate, too, Cdt1 stability, and in this case, specifically during S phase.

Cdt1 has a PCNA-interaction protein motif in its N terminus (PIP motif: Qxxhxxaa) (Warbrick, 2000; Maga and Hubscher, 2003), which is conserved from *C. elegans* to mammalian cells (Arias and Walter, 2006) (Table 1). As described before, mutations that abrogate Skp2 binding cannot completely stabilize human Cdt1 during S phase. Interestingly, additional mutations in the PIP motif do succeed in blocking S phase Cdt1 degradation. Silencing of Cul4 or DDB1 by siRNA also stabilizes mutant Cdt1 protein deficient in Skp2 binding (Hu and Xiong, 2006; Nishitani et al., 2006; Senga et al., 2006). Thus, proteolytic regulation of human

Cdt1 during S phase is carried out by two redundant pathways, involving SCF^{Skp2} and Cul4-DDB1 mediated ubiquitination and degradation (Fig. 4).

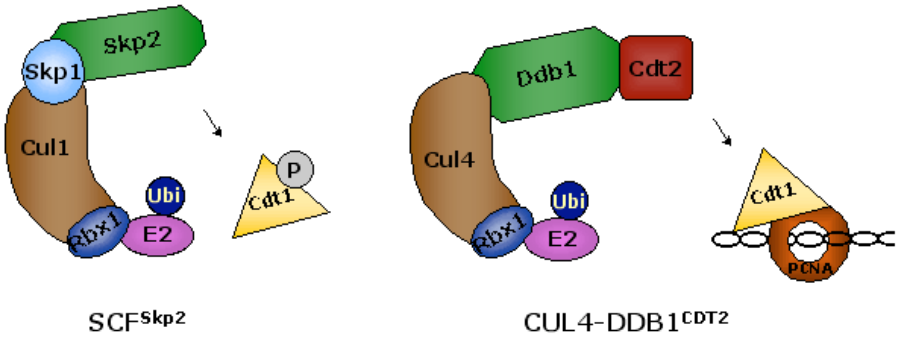

Figure 4: Ubiquitin ligase complexes involved in *Hs*Cdt1 degradation pathways.
On the left, SCF^{Skp2} ubiquitin ligase complex modular structure. SCF^{Skp2} targets Cdt1 for degradation after Cdk/Cyclin complexes phosphorylate Cdt1. On the right, $CUL4\text{-}DBB1^{CDT2}$ ubiquitin ligase complex. Cul4 targets Cdt1 for degradation after Cdt1 binds to PCNA on chromatin during S phase of the cell cycle.

PCNA is also loaded onto chromatin during repair of damaged DNA that operates outside of S phase. In human cells, Cdt1 is also targeted for proteasome-mediated degradation after DNA damage by UV irradiation, and this proteolysis involves ubiquitination of Cdt1 by Cul4-DDB1 ligase (Higa et al., 2003; Hu et al., 2004) and depends on PCNA-Cdt1 interaction (Hu and Xiong, 2006; Nishitani et al., 2006; Senga et al., 2006). It is not very well understood how Cdt1 removal after DNA damage would contribute to blockage of S-phase entry (as discussed by Fujita, 2006), so the biological significance of this mechanism for Cdt1 control is unclear.

	Q-x-x-h-x-x-a-a	aa position
H. sapiens	**Q**-R-R-**V**-T-D-**F**-**F**	3-10
M. musculus	**Q**-S-R-**V**-T-D-**F**-**Y**	3-10
D. melanogaster	**Q**-P-S-**V**-A-A-**F**-**F**	3-10
C. elegans	**Q**-T-A-**V**-T-D-**F**-**F**	14-21
D. rerio	**Q**-A-R-**V**-T-D-**Y**-**F**	3-10
G. gallus	**Q**-L-R-**L**-T-D-**F**-**F**	3-10
X. laevis	**Q**-M-R-**V**-T-D-**F**-**F**	6-13

Table 1: PCNA-interaction protein (PIP) box in Cdt1 homologues.
In the consensus PIP box motif, 'h' represents a hydrophobic aminoacid, 'a' an aromatic aminoacid and 'x' any aminoacid. The numbers in the right represent the amino acid position within the protein.

Arabidopsis conserves several classes of E3 ubiquitin ligases that play important functions during plant cell growth, differentiation and response to the environment (Stone and Callis, 2007). Among cullins, Cul2 and Cul5 are only present in metazoans, while the Cul1, Cul3 and Cul4 members are conserved in all eukaryotes (Thomann et al., 2005). The possible involvement of SCF CUL1- and/or CUL4-based

complexes in CDT1 turnover in Arabidopsis has not been addressed so far. Our studies on the Arabidopsis strategy for Cdt1 control will be addressed in Chapter 4 of this Thesis.

2.3.5 Cdt1 inhibition by geminin

The discovery of **geminin** in metazoans as an inhibitor of pre-RC formation is considered the last redundant level of Cdt1 regulation. Geminin was discovered in a screen for complementary DNA encoding proteins selectively degraded by extracts prepared from mitotic but not interphase Xenopus eggs (McGarry and Kirschner, 1998). Following this initial finding, homologues of Xenopus geminin were characterized in most eukaryotes, including human (McGarry and Kirschner, 1998), Drosophila (Quinn et al., 2001), mouse (Yanagi et al., 2002), Medaka fish (Del Bene et al., 2004) and *C. elegans* (Yanagi et al., 2005), but so far it has not been found in yeast nor plants.

Geminin is a small protein of ~33 kDa with defined structural features (reviewed in Kroll, 2007):

- an N-terminal head that includes a destruction box and a nuclear localization signal;
- a central portion that accounts for an atypical leucine-zipper coiled-coil, necessary for homodimerization and formation of a tetramer super structure (Okorokov et al., 2004) and for the formation of the two separate Cdt1 interaction interfaces (Benjamin et al., 2004; Lee et al., 2004; Saxena et al., 2004);
- a C-terminal tail with no recognizable domains.

Geminin acts by preventing the loading of Mcm proteins onto chromatin without interfering with ORC and Cdc6 association, what suggested a function over Cdt1 (McGarry and Kirschner, 1998). This suggestion was early proven. Human geminin was found to associate with *Hs*Cdt1 producing the inhibition of the pre-RC assembly, an inhibition that could be reversed by addition of an excess of *Hs*Cdt1 to Xenopus egg extracts (Wohlschlegel et al., 2000). Since then, a lot of data has been published supporting the role of geminin in the prevention of re-replication by inhibiting Cdt1 function after entry into S-phase (McGarry and Kirschner, 1998; Wohlschlegel et al., 2000; Tada et al., 2001). High levels of geminin appear as S phase proceeds and continue to accumulate until late M-phase when it is degraded in an APC-dependent reaction (McGarry and Kirschner, 1998), allowing the licensing of the origins for the next cell cycle.

Geminin/Cdt1 interaction does not only inhibit Cdt1 function, but also protects Cdt1 from proteasome-mediated degradation by inhibiting its ubiquitination (Ballabeni et al., 2004). Geminin is, in this way, ensuring basal levels of CDT1 during S phase and its accumulation during mitosis. Consistently, inhibition of geminin synthesis during M phase leads to impairment of pre-RC formation and DNA replication during the following cell cycle (Ballabeni et al., 2004).

Geminin orthologues have not been found in unicellular organisms, including yeast, nor in multicellular non-metazoan organisms, like plants, suggesting that geminin is likely to be specific at least to some forms of multicellular metazoans (Caro and Gutierrez, 2007). This is supported by the previous observation that inhibiting Cdk activity can stimulate re-replication in yeast (which lack geminin) but not in metazoan (which contain geminin as an additional safeguard) (Correa-Bordes and Nurse, 1995; Dahmann et al., 1995; Sun et al., 2000). In metazoans, when Cdk activity is experimentally decreased at the end of the S-phase, cells are again reset for replication, not leading to re-replication, but resulting in endopolyploidy (Hayles et al., 1994; Moreno and Nurse, 1994; Itzhaki et al., 1997). This principle is curiously conserved also in plants, where a reduction in CDK activity is required for cells to endoreplicate (Verkest et al., 2005) although they do not seem to have a geminin homologue in their genomes.

However, assuring genome integrity after DNA replication is not the only known function of geminin. It is tempting to speculate that in higher eukaryotes, the presence of redundant mechanisms for regulating the fidelity of DNA replication favoured geminin's acquiring additional regulatory roles (as discussed in Kroll, 2007). It is interesting to note that geminin was concurrently identified in a functional expression screen in *Xenopus laevis* embryos for molecules that could expand the early neural plate (Kroll et al., 1998). Now we know that, in both vertebrate and invertebrate embryos, geminin plays a critical role in neural cell fate determination. This aspect will be addressed in Chapter 2 of this Thesis, discussing the functional analogies of geminin and a plant Cdt1-interacting protein (GEM) identified in the course of this work (Caro et al., 2007) and their involvement in development and organogenesis.

3. Cell cycle in the context of a whole organism: development

When studying development of multicellular organisms it is more appropriate to consider the concept of cell proliferation than that of cell cycle or cell division itself.

Cell proliferation includes cell cycle progression, cell cycle arrest and re-entry, endoreplication, exit to cell differentiation and cell death, acting together and coordinately since, in multicellular organisms, cell division has to be coordinated with organ growth and specific developmental programmes.

An emerging view is that cell cycle regulatory components, in addition to controlling cell cycle progression, also have important roles in controlling cell proliferation in the context of a developing organism. Altering cell cycle control has profound consequences in organogenesis, although plants seem to be very tolerant to changes in the level of cell cycle regulators (Gutierrez, 2005; De Veylder et al., 2007), and disturbances in cell proliferation are not associated with programmed cell death or oncogenic transformation, as it occurs in animals. The control of cell proliferation and differentiation during plant development depends, also, on the concerted action of different hormones. Among them, auxins and cytokinins are the ones better known to impinge directly on cell cycle regulators (Stals and Inzé, 2001; Hartig and Beck, 2006). In addition, other hormones, e.g. abscisic acid, ethylene, jasmonic acid and brassinosteroids, whose action is less well characterized, also have a proved impact on cell cycle progression and/or arrest (del Pozo et al., 2005).

3.1 Proliferation/Differentiation balance

Transitions between cellular programs regulating cell proliferation and cell differentiation must be precisely coordinated and temporally controlled during development, to ensure that a proper number of cells form the correct patterns in tissues and organs. But even before a cell differentiates, it is absolutely necessary that it adopts the correct cellular identity. This is why coordination of cell proliferation with cell fate decisions is at the basis of any developmental process.

3.1.1 Metazoan organisms

Development depends critically on the intricate balance between proliferation and differentiation. Even though the signals controlling the proliferation-differentiation balance *in vivo* remain elusive, some molecules have so far been proposed to have a role in this transition. Interferon regulatory factor 6 in epidermis development (Richardson et al., 2006), estrogen receptor α in differentiation of neural cell progenitors (Ciana et al., 2003), Sonic hedgehog during mouse lung organogenesis (Li et al., 2004) and N-Myc during neuronal differentiation (Ciani et al., 2004) are some examples of molecules involved in this proliferation/differentiation switch.

Apart from its role in regulating DNA replication, **geminin** has an additional function that has recently been uncovered. It is a well-known molecular coordinator of cell proliferation, fate and differentiation during embryonic metazoan development. In almost all cellular contexts defined to date, geminin expression correlates strongly with actively dividing, progenitor cell states in the embryo and in the adult, while being down-regulated prior to or coincident with cell cycle arrest (Quinn et al., 2001; Wohlschlegel et al., 2002; Xouri et al., 2004; Montanari et al., 2005). Geminin also correlates with proliferating cells in many cancers, marking aggressive neoplasms since it specifically labels cells with an increased rate of cell cycle progression and shortened G1 phase (Wohlschlegel et al., 2002; Gonzalez et al., 2004). Many studies have proven geminin requirement for regulating **vertebrate** embryonic development. In *Xenopus laevis*, geminin can regulate neural cell fate at gastrulation through its N-terminal domain (Kroll et al., 1998; McGarry, 2002). Geminin's C-terminal domain has been shown to regulate later neurogenesis, when neuronal precursors exit the cell cycle and differentiate by antagonizing Brg1 activity (a catalytic subunit of the SWI/SNF chromatin remodelling complex) (Seo et al., 2005).

A role for geminin has also been postulated in controlling *Hox* gene expression and activity. Vertebrate *Hox* genes are arranged in four clusters and are expressed in overlapping patterns along the anterior-posterior axis of the embryo. After establishment of *Hox* expression patterns, these are maintained through many subsequent cell divisions by the Trithorax Group and Polycomb Group proteins (Gebuhr et al., 2000; Ringrose and Paro, 2004). Geminin can associate directly with Hox proteins and with the Polycomb Group protein Scmh1 during chick embryogenesis (Luo et al., 2004), negatively impacting on *Hox* function and expression to regulate antero-posterior axial patterning (Luo et al., 2004; Saxena et al., 2004). Geminin can also block homeobox gene expression in Medaka retinogenesis, where it binds to and antagonizes the function of the homeodomain protein Six3 (Del Bene et al., 2004).

Another potential developmental role for geminin is in the **invertebrate** Drosophila eye (Quinn et al., 2001), resembling again the activity of vertebrate geminin homologues. Gain and loss of function of Drosophila geminin in the nervous system support roles in regulating neuronal cell fate and neural differentiation (Quinn et al., 2001).

Together, these data define common features of geminin activity in various

developmental contexts as a coordinator of cell cycle and transcriptional regulatory pathways that control cell fate decisions and differentiation during organogenesis (reviewed in Kroll, 2007). In Chapter 2 of this Thesis, I will present a comparative evaluation of the role played by geminin in animals and by GEM in plants, two apparently unrelated proteins that, nevertheless, possess a series of intriguing analogies through their participation in cell division control and locus-specific changes in epigenetic marks during development (Caro and Gutierrez, 2007).

3.1.2 Plants

To understand development it is useful to analyze simple systems. Arabidopsis is widely used by plant molecular geneticists to study both basic and applied problems. Unlike animals, plants develop continuously and in response to their environment. This developmental plasticity resides, at least partly, in that plant organs are constantly produced from pools of proliferating cells which are found at the meristems. Different models have been used in Arabidopsis to study cell proliferation in the context of development: embryonic development (Willemsen and Scheres, 2004), the formation of organs from the shoot apical meristem (Carles and Fletcher, 2003), the floral meristem (Lohmann and Weigel, 2002) or the root meristem (Benfey and Scheres, 2000), mainly. Because of the simplicity of its organization, we have chosen the Arabidopsis root as a model to study the coordination of cell proliferation and differentiation during development.

Arabidopsis roots can be viewed as a set of concentric cylinders (reviewed in Benfey and Scheres, 2000). The four outer layers, the epidermis, cortex, endodermis and pericycle surround the vascular tissue located in the inner part of the root. The outer epidermis is composed of two cell types, those that form root hairs (hair cells) and those that do not (non-hair cells). The cortex and endodermis layers are each composed of a single cell type, and include almost invariably eight cells in each layer. The pericycle is made up of cells that can initiate the formation of new lateral roots, and in the center of the root, we can find the vascular tissue (Fig. 5). Within the growing root, new cells originate at the distal tip in a region known as the meristem. Sets of initials (the functional equivalent of animal stem cells) are located around a non-dividing set of cells, which form the quiescent center (QC).

The QC is the source of a signal that maintains the undifferentiated state of the stem cells. Each set of initials goes through a stereotyped division pattern to generate its progeny. Because plant cells do not move in relation to each other, the

divisions of the root initials give rise to columns or files of cells. The spatial relationship of cells in a file reflects their age. Younger cells are near the root tip, older cells are higher up in the root. Therefore, all developmental stages are present in every root and anatomy reflects ontogeny (Benfey and Scheres, 2000).

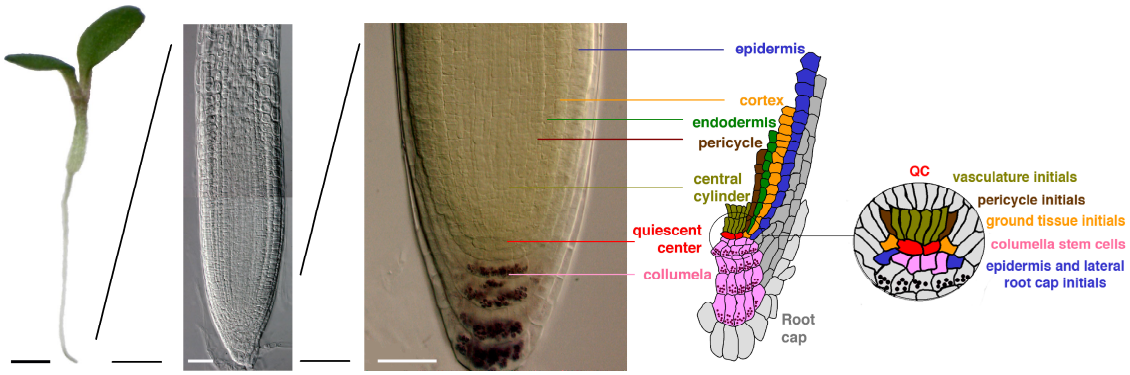

Figure 5: Images and schematic representation of the Arabidopsis root meristem.
From left to right, an Arabidopsis seedling (scale bar, 0.5 mm), a root mounted with chloralhydrate (scale bar, 40 μm) and a lugol-stained root in which starch granules of columella cells appear coloured in violet (scale bar, 40 μm). On the right, a scheme showing the different cell types and their organization in the root meristem and a detail of the quiescent center (QC) and initial stem cells that surround it.

A balance between cell production and cell differentiation must be achieved by controlling the rate of cell division in meristematic cells. If such control is perturbed, the meristem may not maintain itself or be abnormally enlarged. The role of GEM in controlling the coordination between cell fate specification and different kinds of cell divisions in the root, necessary to produce a proper organ pattern, will be discussed in depth in the Chapter 3 of this Thesis.

Because the root epidermis is readily accessible and consists of only two cell types, it has become a well-studied model for cell differentiation and cell patterning in plants. The arrangement of hair and non-hair cell types within the epidermis varies in different plant species (reviewed in Schiefelbein et al., 1997). In some plant species, there is no apparent pattern of root hair and hairless cells. In others, including many monocots, epidermal cell fate is linked to an asymmetric cell division, with the smaller daughter cell differentiating into a root hair cell and the larger daughter cell generating one or more mature hairless cells. In a third group of plants, which includes Arabidopsis and other members of the *Brassicaceae*, a position-dependent pattern of epidermal cell types is generated. In this case, meristematic epidermal cells in contact with two underlying cortical cells ("T" position) adopt trichoblast identity and will differentiate into root hair cells, whereas cells over just one cortical cell ("A" position) will adopt atrichoblast fate and will differentiate into non-hair cells (Fig. 6).

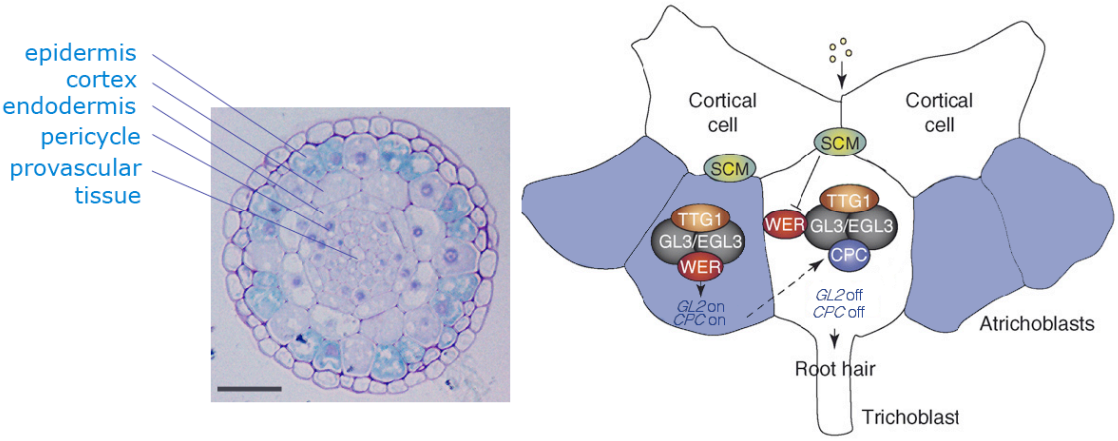

Figure 6: Cross section of an Arabidopsis root meristem (left, scale bar 25 μm) and scheme of the transcriptional network that controls *GL2* and *CPC* expression in the epidermis of Arabidopsis root meristem to determine trichoblast and atrichoblast cell fate (right).

The non-hair cell type arises in cell files located at the "A" position and requires the activity of the homeodomain protein GLABRA2 (GL2) (Rerie et al., 1994; Masucci et al., 1996), the WD40-repeat protein TRANSPARENT TESTA GLABRA 1 (TTG1) (Galway et al., 1994; Larkin et al., 1999), the two-repeat R2R3 MYB protein WEREWOLF (WER) (Lee and Schiefelbein, 1999), and the two related basic helix–loop–helix (bHLH) proteins GLABRA3 (GL3) and ENHANCER OF GLABRA3 (EGL3) (Bernhardt et al., 2003). The GL3 and EGL3 proteins physically interact with WER and TTG1 proteins, and each of these is required for *GL2* transcription at the A cell position, which implies that a WER-GL3/EGL3-TTG1 transcription complex positionally regulates the non-hair cell fate (Hung and Hultgren, 1998; Lee and Schiefelbein, 1999; Payne et al., 2000; Bernhardt et al., 2003) (Fig. 6).

The hair cell type is specified in epidermal cell files located outside a longitudinal cortical cell junction (the "T" position) by the action of three related single-repeat R3 MYB proteins, CAPRICE (CPC), TRIPTYCHON (TRY), and ENHANCER OF TRY AND CPC1 (ETC1) (Wada et al., 1997; Schellmann et al., 2002; Kirik et al., 2004). The CPC, TRY, and ETC1 proteins have been proposed to promote the hair cell fate by displacing WER from the WER-GL3/EGL3-TTG1 complex in the T cell position (Dolan and Costa, 2001; Schellmann et al., 2007). The CPC protein is capable of moving from the A cells to the T cells (Wada et al., 2002; Kurata et al., 2005), what implies that CPC (and perhaps the other single-repeat MYBs) acts by a lateral inhibition mechanism to specify the hair cell pattern (Lee and Schiefelbein, 2002; Schellmann et al., 2007) (Fig. 6). Recently, a receptor-like kinase, encoded by a gene *SCRAMBLED* (*SCM*) (Fig. 6), has been reported to mediate the positional signaling and the expression patterns of the *CPC*, *GL2*, and *WER* genes (Kwak et al., 2005).

However, information about whether the *SCM* gene interacts with the known patterning genes as well as the mechanism used, is still lacking.

Xu et al. (2005) showed that histone acetylation has a mechanistic role in regulating the position-dependent expression of patterning genes in the Arabidopsis root epidermis. Later, Costa and Shaw (2006) showed that alternative states of chromatin organization around the *GL2* locus are required to control position-dependent cell-type and that chromatin status around the *GL2* locus is reorganized each cell cycle, what determines whether *GL2* can be transcribed in daughter cells or not. The involvement of a novel protein identified in this study (GEM) in controlling epidermal cell division potential and histone modifications in the promoters of the patterning genes *GL2* and *CPC* will be addressed in Chapter 1 of this Thesis. Future studies should aim at identifying the molecular basis of the coordination between cell division, cell fate decisions and cell differentiation. This kind of analysis show how comparative studies of basic processes in model animals and plants are enlightening for our understanding of organogenesis in multicellular organisms.

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Objectives

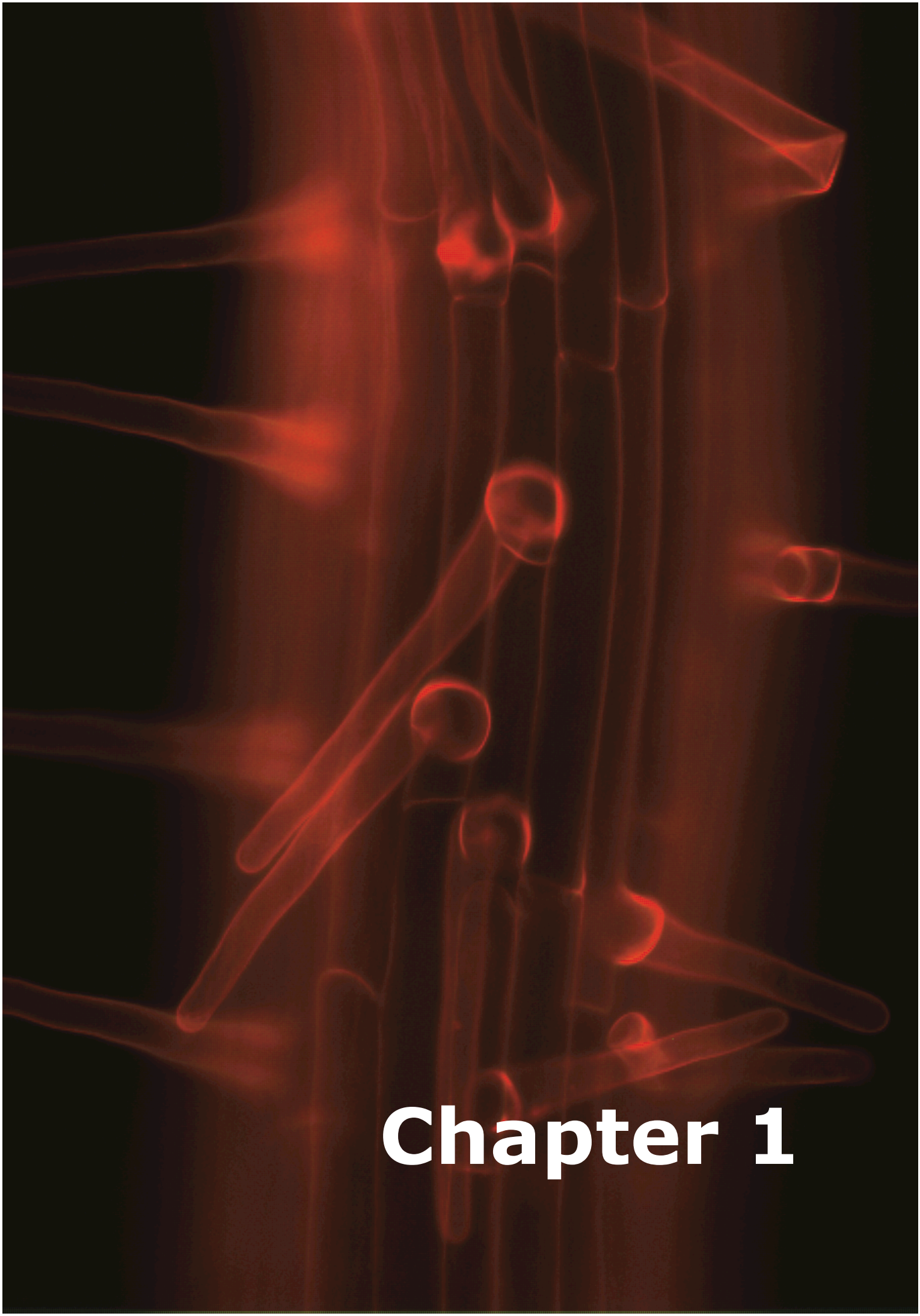
The main objectives of this Thesis work are to:

- Identify novel factors interacting with proteins of the pre-Replicative Complex of *Arabidopsis thaliana*.

- Study the implication of the pre-Replicative Complex and related proteins in the control of cell division and endoreplication in the context of a developing organism.

- Assess the involvement of pre-Replicative Complex and related proteins in cell fate decisions and differentiation during organogenesis.

- Define the strategies controlling cell proliferation/cell differentiation balance in metazoan and plant systems.


Chapter 1

Root hairs in an Arabidopsis GEM^{OE} root stained with propidium iodide

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Chapter 1:

A chromatin link that couples cell division to root epidermis patterning in Arabidopsis

A chromatin link that couples cell division to root epidermis patterning in Arabidopsis

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Abstract

Cell proliferation and cell fate decisions are strictly coupled processes during plant embryogenesis and organogenesis (Blilou et al., 2002; Fletcher, 2002; Gutierrez, 2005; Jenik et al., 2005; Wildwater et al., 2005). In the *Arabidopsis thaliana* root epidermis, expression of the homeobox *GLABRA2* (*GL2*) gene determines hair/non-hair cell fate (Di Cristina et al., 1996; Masucci et al., 1996). This requires signaling of positional information from the underlying cortical layer (Dolan et al., 1993; Kwak et al., 2005), complex transcriptional regulation (Larkin et al., 2003; Guimil and Dunand, 2006) and a change in chromatin accessibility (Costa and Shaw, 2006). However, the molecular connections among these factors and with cell division are not known. Here we have identified a *GL2*-expression modulator, GEM, as an interactor of CDT1, a DNA replication protein. GEM also interacts with TTG1 (TRANSPARENT TESTA GLABRA1), a WD40-repeat protein involved in *GL2*-dependent cell fate decision, and modulates both cell division and *GL2* expression. Here we show that GEM participates in the maintenance of the repressor histone H3K9 methylation status of root patterning genes, providing a link between cell division, fate and differentiation during Arabidopsis root development.

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Results and Discussion

Root epidermal cell fate decisions are triggered by a positional cue delivered by the cortical cell layer. Then, cell fate fixation and differentiation depends on a complex transcription factor network that regulates the expression of the *GLABRA2* (*GL2*) gene. *GL2* is expressed in atrichoblasts—epidermal cells in contact with a single cortical cell that do not produce root hairs. On the contrary, in the trichoblasts, which will differentiate into root hair cells and are in contact with two cortical cells, *GL2* is not expressed (Di Cristina et al., 1996; Masucci et al., 1996; Larkin et al., 2003; Serna, 2004). Cell fate specification during epidermal root patterning is also affected by cell division (Berger et al., 1998). Thus, radial symmetry depends on the occurrence of divisions that generate a characteristic pattern of tricho- and atrichoblast files in the epidermis (Supplementary Fig. 1).

The initial hint of a coupling between cell division and fate came from the finding that Arabidopsis plants overexpressing CDT1, a component of the pre-replication complexes that controls initiation of DNA replication in eukaryotes (DePamphilis et al., 2006), have increases in both cell division potential (Castellano et al., 2004) and *GL2* messenger RNA levels (Fig. 1a). An altered *GL2* expression was not observed in *CDC6*^{OE} plants (Fig. 1a). Yeast two-hybrid screenings using the two CDT1 proteins encoded by the Arabidopsis genome retrieved a complementary DNA clone (Fig. 1b) that encodes a previously unidentified protein without significant homology to any known entry in the databases, which did not interact with CDC6 (not shown). It was named GEM (*GL2* expression modulator, see below).

Real-time PCR with reverse transcription (RT–PCR) revealed that the *GEM* gene is expressed ubiquitously in the plant and in all root cells (Supplementary Fig. 2). To define the function of GEM, we selected homozygous lines of a T-DNA insertion mutant with a ~5-fold reduction in full-length *GEM* mRNA levels (*gem-1*) and generated plants overexpressing *GEM* (*GEM*^{OE}) with constitutively increased (~6-fold) *GEM* mRNA and protein levels (Fig. 1c and Supplementary Fig. 3). *GL2* mRNA levels inversely correlate with *GEM* expression (Fig. 1c). Root hair density was reduced in *gem-1* plants and increased in *GEM*^{OE} plants (Fig. 1d), a phenotype which is already detectable when hairs initiate differentiation (Supplementary Fig. 4), indicating that GEM acts early in hair specification and/or differentiation. In leaves, *GL2*-expressing epidermal cells are specified as trichomes (Larkin et al., 2003; Serna, 2004). Consistent with a participation of GEM in *GL2*-mediated epidermal cell fate decisions, trichome density was increased in *gem-1* and reduced in *GEM*^{OE} plants (Fig. 1e).

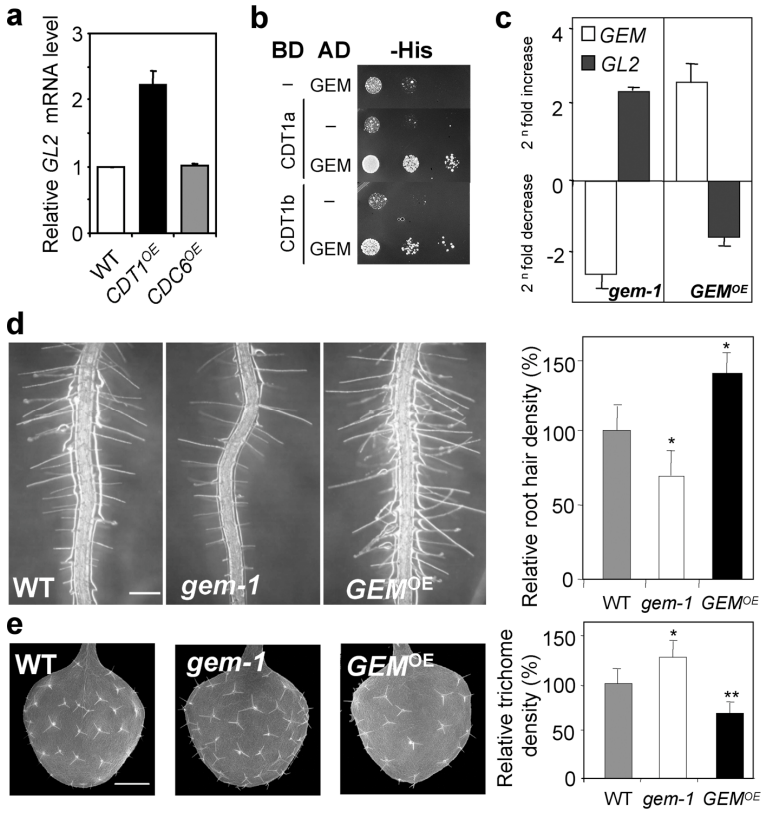


Figure 1: Identification of GEM, and root hair and trichome phenotypes in mutant plants.
a) Expression of the homeobox *GL2* gene measured by real-time RT–PCR in *CDT1*^{OE} or *CDC6*^{OE} seedlings (10 days old). Values represent mean ± s.d. (n=3).
b) Isolation of GEM as a CDT1-interactor in yeast two-hybrid screenings using CDT1 and GEM proteins fused to the GAL4 binding domain (BD) and activation domain (AD), respectively.
c) Determination of *GEM* and *GL2* mRNA levels (see Methods). Measurements were made relative to the wild type (WT) and values represent mean ± s.d. (n=3).
d) Root hair phenotype of *gem-1* and *GEM*^{OE} plants. Phase-contrast microscopy images of the mature part of the rotos (scale bar 150 μm). Quantification of the root density (mean ± s.d.; n=5 roots) is shown at the right. Asterisks indicate statistically significant differences (*P<0.1).
e) Trichome phenotype of *gem-1* and *GEM*^{OE} plants. Scanning electron microscopy images of the adaxial surface of leaves (leaf number 1/2, 8 days old; scale bar 1 mm). Quantification of trichome density (mean ± s.d.; n=10 leaves) is shown at the right. Asterisks indicate statistically significant differences (**P<0.05; *P<0.1).

Plants expressing the GUS reporter gene under the control of the *GL2* promoter (*pGL2*-GUS (Masucci et al., 1996)) in the different GEM backgrounds revealed a role of GEM in the spatial patterning of *GL2* expression. The epidermis of mutant plants also showed alterations of the number and pattern of cell files (Fig. 2a). Quantification of epidermal clones indicated that *gem-1* plants showed a ~2-fold increase in the frequency of longitudinal (anticlinal) cell divisions, whereas the opposite occurred in the GEM^{OE} plants (Fig. 2b). This was also the case in the CDT1^{OE}, but not in the CDC6^{OE}, plants (Supplementary Fig. 5).

Transverse sections of the root meristem revealed that GEM restricts cell division potential in both epidermal and cortical cells in *gem-1* roots (Fig. 3a, b). In addition, *GL2* was occasionally misexpressed in the *gem-1* and GEM^{OE} plants (Fig. 3a), which showed a significant increase of ectopic atrichoblasts and trichoblasts, respectively (Fig. 3c; Supplementary Fig. 6). The *gem-1* GEM^{OE} plants showed a phenotype similar to that of GEM^{OE} plants (not shown). Together, these experiments establish that GEM restricts cell division and negatively regulates *GL2* expression, strongly suggesting that GEM is a component of the pathway that promotes root hair specification and differentiation.

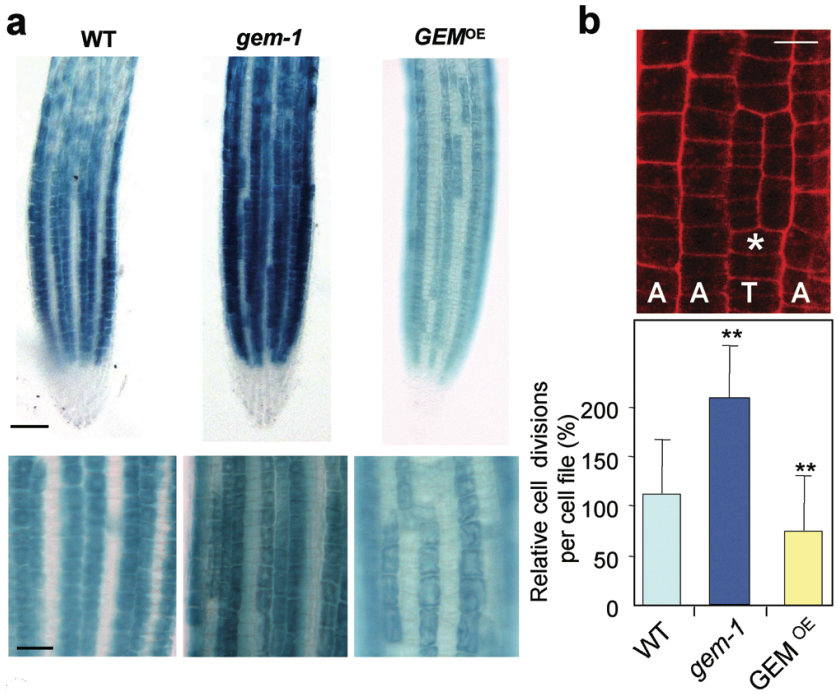


Figure 2: GEM regulates the expression of the cell fate *GL2* gene.
a) Expression of the p*GL2*-GUS reporter gene in 5-day-old seedlings (upper panels; scale bar 50 μm). Also note the alterations in cell file pattern in mutant plants, at higher magnification (lower panels; scale bar 25 μm).
b) GEM negatively affects cell division in the root epidermis. Upper panel shows an example of an epidermal clone (asterisk). The trichoblast (T) and atrichoblast files (A) are indicated. Cell walls were visualized with propidium iodide (scale bar 10 μm). Lower panel, quantification of the frequency of cell clones per file in the indicated plants. Values are mean ± s.e.m. (n=3 experiments; **P<0.05).

There is the possibility that GEM regulates *GL2* expression through direct interaction with its promoter DNA. This hypothesis was discarded because a DNA binding domain is not predicted in GEM and it did not form specific complexes either with a *GL2* promoter probe or with random DNA sequences (not shown). Another possibility is that GEM may alter the expression of *GL2* expression regulators.

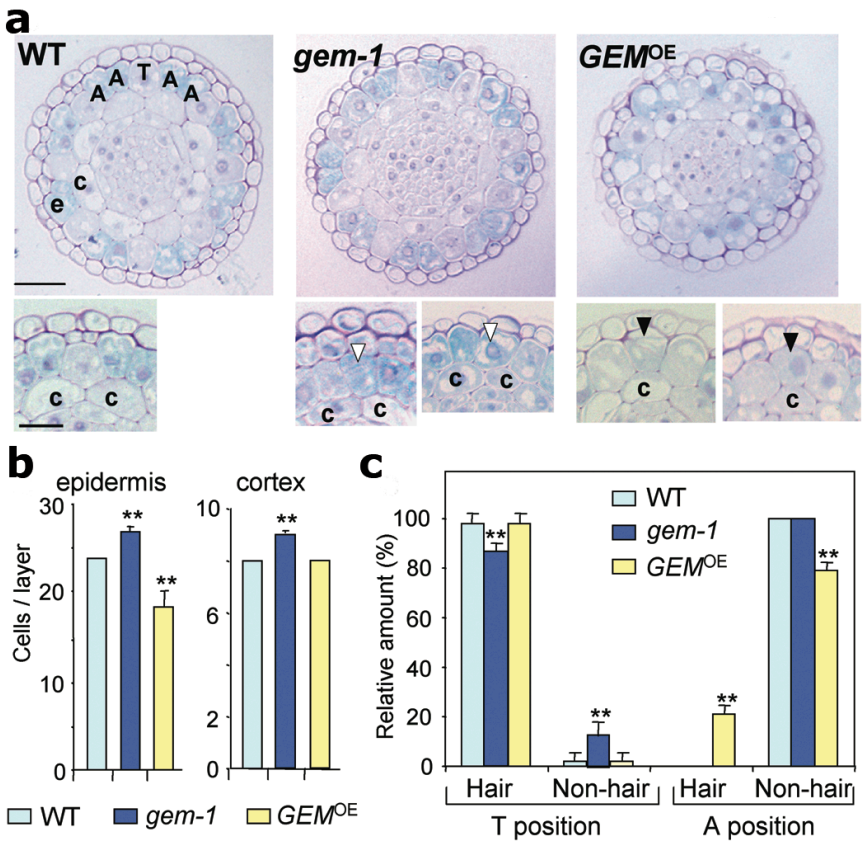


Figure 3: GEM regulates the epidermal cell division rate and cell fate.
a) Transverse sections of root meristems (~200–250 μm from the tip) of plants expressing the *pGL2*-GUS marker. The outermost cell layer is the root cap. In the larger panels, note the altered cell file organization of the cortical (c) and epidermal (e) cell layers in *gem-1* and *GEM*^{OE} plants (scale bar 25 μm). In the smaller panels, note the ectopic *GL2* expression in *gem-1* plants (white arrowheads) and the reduced *GL2* expression in the *GEM*^{OE} plants (black arrowheads). Scale bar 10 μm.
b) Epidermal and cortical cell number quantified in transverse sections (200 μm from the root tip). Values are mean ± s.d. (n=4; **P<0.05).
c) Quantification of hair and non-hair cells in trichoblast (T) and atrichoblast (A) positions (see Supplementary Fig. 6 for details). Asterisks indicate statistically significant differences (**P<0.05).

A complex containing TTG1, the bHLH factors GL3 (GLABRA3) and EGL3 (ENHANCER OF GLABRA3), and the R2R3 Myb factor WER (WEREWOLF), binds to and activates the *GL2* promoter in atrichoblasts (Larkin et al., 2003; Bernhardt et al., 2005; Guimil and Dunand, 2006). Expression of *CAPRICE* (CPC), which inactivates the function of WER (Kurata et al., 2005), is also under the control of the TTG1–GL3–EGL3–WER complex (Ryu et al., 2005). *CPC* expression parallels that of *GL2*, being increased in *gem-1* and reduced in *GEM*^{OE} plants (Fig. 4a), whereas mRNA levels of *TTG1, GL3* and *EGL3* showed small changes that did not correlate with *GEM* levels (Fig. 4a). *WER* mRNA levels in *gem-1* and *GEM*^{OE} plants were the opposite to that of *GL2* and *CPC* (Fig. 4a).

As expected, the expression of all these genes in *CDT1*^{OE} plants followed a pattern similar to that in *gem-1* plants, but these effects were not due to changes in *GEM* expression (Supplementary Fig. 7). Thus, the effect of GEM on *GL2* and *CPC* expression is not due to changes in the expression level of other components of the transcriptional regulatory complex. A third possibility is that GEM functions by interacting directly with components of the TTG1–GL3–EGL3–WER/CPC multimeric complex. Yeast two-hybrid assays indicated that TTG1 was the only one that physically interacted with GEM (not shown). Pull-down experiments also demonstrated that partial deletion of the WD40 repeats of TTG1 decreased the GEM–TTG1 interaction (Fig. 4b), and that the amino-terminal moiety of GEM is sufficient for TTG1 binding (Supplementary Fig. 8). A yeast three-hybrid assay showed that TTG1 can disrupt the CDT1–GEM interaction (Fig. 4c), suggesting that competition of GEM for CDT1 and TTG1 is crucial for both cell division control and *GL2* and *CPC* expression. Chromatin immunoprecipitation (ChIP) experiments revealed that GEM is recruited specifically to the *GL2* and *CPC* promoters (Fig. 4d).

We also analyzed the genetic interactions of *GEM* with *TTG1* and *SCRAMBLED* (*SCM*) (Kwak et al., 2005), also known as *STRUBBELIG* (*STB*) (Chevalier et al., 2005), which encodes a receptor-like kinase required to interpret positional signals during epidermal cell fate specification. The *ttg1-1* null mutation, which results in ectopic trichoblasts (Larkin et al., 2003; Serna, 2004), rescued the decreased hair density phenotype of *gem-1* and prevented the appearance of ectopic atrichoblasts (Fig. 4e). We found that the *scm-2* mutation, which produces ectopic atrichoblasts but does not affect hair density, did not rescue the increased hair density phenotype of *GEM*^{OE} plants (Fig. 4e). Also, the presence of ectopic atrichoblasts, which occur in *scm-2* roots, but not in *GEM*^{OE}, was maintained in the *scm-2 GEM*^{OE} roots (Fig. 4e). Therefore, we conclude that GEM is part of the complex that represses *GL2* and *CPC* expression through TTG1, and that GEM and SCM act, at least in part, in different pathways.

Global changes in histone acetylation affect the expression of root-patterning genes (Xu et al., 2005). Thus, we tested whether GEM affects the histone modification status that ultimately controls *GL2* and *CPC* expression. ChIP experiments indicated that histone H4 acetylation did not change in response to alterations of GEM (Fig. 5a). However, both promoters contained histone H3K9acK14ac in *gem-1* plants, whereas this mark was absent in *GEM*^{OE} plants (Fig. 5a), consistent with GEM behaving as a negative regulator of gene expression.

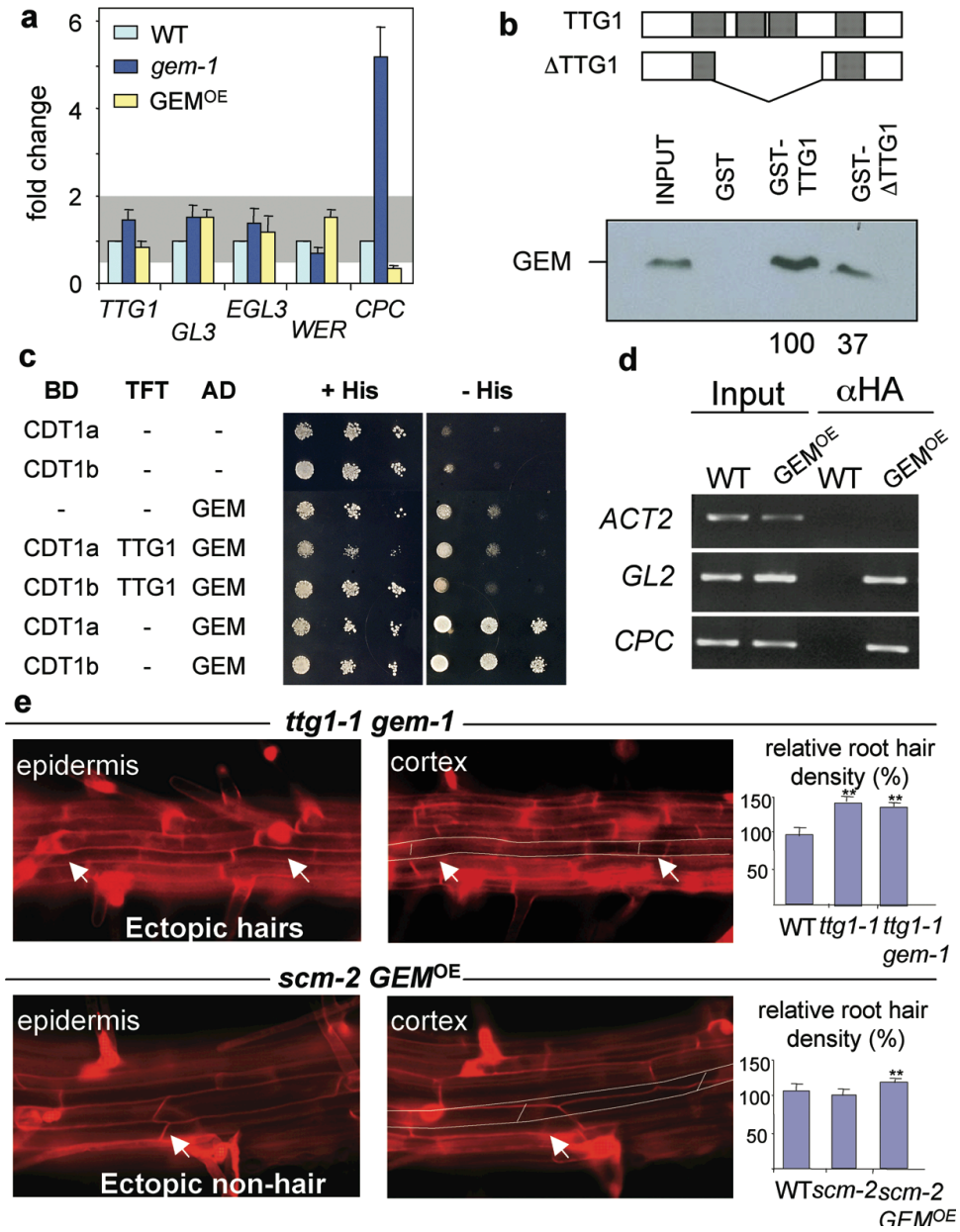


Figure 4: GEM interacts with TTG1, CDT1 and with the *GL2* and *CPC* promoters.
a) Expression of regulators of *GL2* expression determined by realtime RT–PCR in extracts of 10-day-old seedlings. Shaded area represents a twofold increase or decrease. Values represent mean ± s.d. (n=3).
b) Schemes of full-length and truncated TTG1 used in the pull-down experiments. Details are provided in Methods.
c) TTG1 disrupts the CDT1–GEM interaction in a yeast three-hybrid assay (see Methods). BD and AD refer to proteins fused to the GAL4 DNA binding and activation domain, respectively.
d) Chromatin immunoprecipitations were performed with 10-day-old *GEM*^{OE} seedlings to reveal GEM in the *GL2*, *CPC* and *ACTIN2* (*ACT2*) promoters. Input refers to samples before addition of the antibody.
e) Ectopic hairs and non-hairs (arrows), and hair density (mean ± s.d.; n=6; **P<0.05) in the *ttg1-1 gem-1* and *scm-2GEM*^{OE} plants.

The consequences of introducing H3 methylation marks differ between mammals and Arabidopsis. Contrary to the situation in mammalian cells, H3K9me1 and H3K9me2 in Arabidopsis are typical of silent heterochromatin regions, whereas

H3K9me3 associates with active euchromatin (Fischer et al., 2006; Fransz et al., 2006; Fuchs et al., 2006). Both *GL2* and *CPC* promoters contained increased levels of H3K9me3 in *gem-1* plants, but not in *GEM*^{OE} plants, and the reverse occurred for H3K9me2 (Fig. 5a), confirming a role of GEM to be regulating the level of H3K9me3. Scanning each locus revealed that, in both cases, GEM-dependent changes in the histone H3ac and H3K9me marks were located just upstream of the open reading frame (Fig. 5b). Therefore, GEM mediates the acquisition and/or maintenance of the correct histone modifications at these two genes that are responsible for cell fate decisions in the root epidermis.

Two issues deserve special attention. One is that alternative states of accessible and closed chromatin around the *GL2* locus determine position-dependent cell fate specification, and *GL2* expression and cell fate is reset in each cell cycle (Costa and Shaw, 2006). Analysis of *GL2* and *CPC* promoters in synchronized cells revealed that their epigenetic marks are cell-cycle-regulated in a pattern consistent with them being repressed in G2/M cells and active early in the cell cycle (Fig. 5c). This is reminiscent of the DNA replication licensing mechanism, which also operates in late mitosis and early G1 (DePamphilis et al., 2006). The other issue refers to geminin, an inhibitor of the CDT1-mediated chromatin licensing (DePamphilis et al., 2006), identified in animal cells but not in yeast or plants. Geminin also coordinates proliferation and differentiation decisions during animal embryogenesis through various mechanisms, including the interaction with SWI/SNF or Polycomb chromatin complexes (Seo and Kroll, 2006). Thus, whether GEM, identified here as a CDT1-interacting protein that mediates histone H3 modifications and cell fate decisions, is an Arabidopsis functional homologue of animal geminin is an intriguing possibility.

GEM shows a very low amino acid homology with geminin and it lacks the coiled-coil domain. In any case, it is remarkable that regulation of homeobox gene expresión by bHLH proteins in Arabidopsis might use a general strategy similar to that of cell differentiation in animals (Seo and Kroll, 2006). Furthermore, altering geminin levels in mammalian cultured cells leads to partial chromosome re-replication and genomic instability (Melixetian et al., 2004; Zhu et al., 2004). Flow-cytometry analysis of *gem-1* and *GEM*^{OE} plants have so far not revealed significant changes in DNA content (unpublished data); whether the chromatin reorganization that dictates cell fate decisions is associated with the chromatin licensing process is a question to be addressed in the future. Our results are consistent with a model where GEM targets the CDT1- and TTG1-dependent control of cell division and *GL2* and *CPC* expression in the root (Fig. 5d).

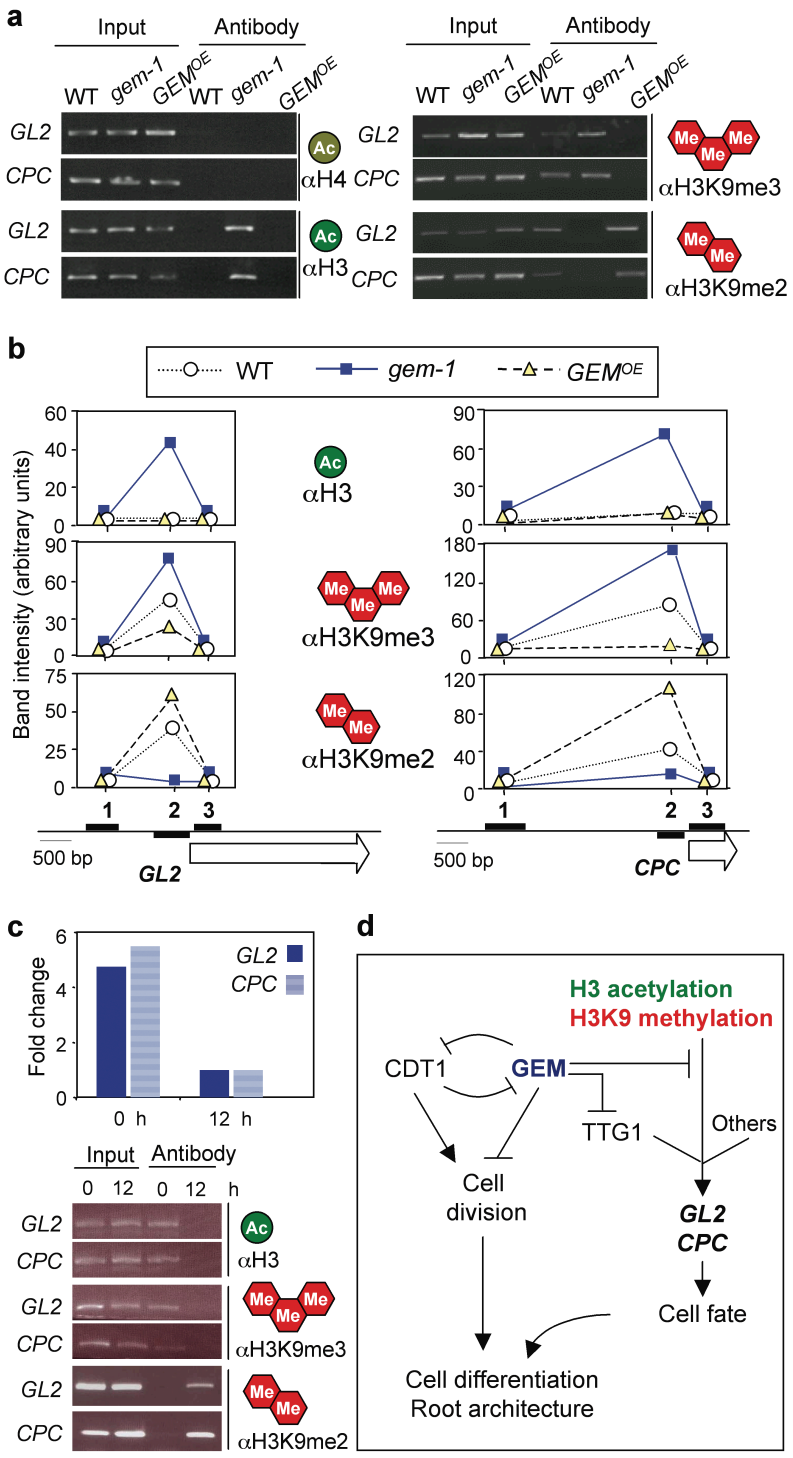


Figure 5: GEM controls the histone H3 acetylation and K9 methylation status of *GL2* and *CPC* genes.
a) Histone modifications at the *GL2* and *CPC* promoters in wild-type, *gem-1* and *GEM*^{OE} plants shown by ChIP. The promoter fragment amplified in each gene corresponds to fragment 2 in panel b.
b) Quantification of histone H3ac, H3K9me3 and H3K9me2 marks around the *GL2* and *CPC* loci. Fragments amplified are indicated in the maps. The data are representative of two independent assays.
c) Epigenetic marks of the *GL2* and *CPC* promoters during the cell cycle. Arabidopsis cultured cells were arrested in G0/G1 by sucrose deprivation (0 h) and allowed to proceed until G2/M (12 h) by sucrose addition. Their position in the cell cycle was assessed with marker genes (Menges et al., 2005). The levels of *GL2* and *CPC* mRNAs (real-time RT–PCR) and the epigenetic histone modifications (ChIP) were determined.
d) A simplified model that accounts for the role of GEM.

Our study has elucidated a mechanism whereby GEM seems to participate in the maintenance of a repressor histone H3 epigenetic status of the *GL2* and *CPC* promoters. Thus, GEM is a crucial component of the spatial control of cell division, patterning and differentiation of Arabidopsis root epidermal cells.

Methods

Plant material

Arabidopsis seedlings (*Col-0* ecotype) were grown in MS salts medium supplemented with 1% sucrose and 1% agar in a 16 h/8 h light/dark regime at 22 ºC. The Arabidopsis T-DNA insertion line SALK_145846 (homozygous lines generated here were named *gem-1*) was obtained from the Arabidopsis Biological Resource Center (Ohio State University). To generate plants expressing the haemagglutinin (HA)-tagged Arabidopsis GEM protein (GEM^{OE}), the GEM cDNA (GenBank accession number, corresponding to the open reading frame At2g22475, is EF490993) was cloned in-frame with the HA epitope into the pBHA plasmid under the control of a 35S cauliflower mosaic virus promoter. Arabidopsis plants were transformed with the *Agrobacterium tumefaciens* C58CRifR strain (Clough and Bent, 1998). Transformed seedlings (T0 generation) were selected on MS agar plates containing 10 μgml^{-1} BASTA and transferred to soil. T2 homozygous plants were selected for further analysis.

Microscopy

Root hair phenotypes were analyzed with a MZ9.5 stereomicroscope (Leica) and an Axioskop2 Plus microscope (Zeiss), and the images were captured with a digital Coolsnap FX camera (Roper Scientific). Longitudinal divisions of root epidermis were analyzed in 8-day-old liquid-grown seedlings after propidium iodide staining (1 $mgml^{-1}$, 1min) using a BioRad Microradiance confocal microscope. For transverse sections, roots were fixed in 13 PBS, 4% paraformaldehyde, 2.5% glutaraldehyde, 0.1% Triton X-100 for 20 min, and then overnight in 1xPBS, 4% paraformaldehyde, at 4 ºC. After dehydration, whole roots were stained with 0.2% eosine in absolute ethanol, embedded in Epon and 1 μm sections were prepared and stained with toluidine blue.

Histochemical detection of GUS activity

Detection of GUS activity was performed using 5-bromo-4-chloro-3-indolyl-β-D-glucuronide as described (Ramirez-Parra et al., 2004).

Yeast two-hybrid screening and assays

For the yeast two-hybrid screening, yeast cells (HF7c strain) were transformed first with plasmids pGBT8-AtCDT1a or pGBT8-AtCDT1b (pGBT8, Clontech Laboratories) and, then, with an Arabidopsis cell suspension cDNA library (Matchmaker cDNA Library, Clontech). Cells were grown for 3–8 days, and the co-transformants selected in minimal medium as previously described (Ramirez-Parra and Gutierrez, 2000). The transformants recovered during this period were checked for growth in the presence of 20–30 mM 3-AT.

Yeast three-hybrid assays

We generated yeast cells (strain HF7c) expressing Arabidopsis CDT1a or CDT1b (as described for the yeast two-hybrid assays), fused to the GAL4 DNA-binding domain of the pBGT8 plasmid (BD column in Fig. 4c). Then, they were co-transformed with a plasmid that expressed Arabidopsis GEM, fused to the GAL activation domain in the pACT2 plasmid (AD column in Fig. 4c). These combinations allowed yeast growth in selective medium (-His), indicating a strong and specific interaction. The assays were carried out using the pTFT1 vector (Egea-Cortines et al., 1999) to express the third protein, as described (Desvoyes et al., 2006). We co-transformed yeast cells with a third plasmid to express TTG1 (TFT column in Fig. 4c). In the absence of TTG1, neither CDT1 (a or b) nor GEM proteins alone allowed yeast cell growth. However, transforming the third plasmid expressing TTG1 largely impaired the strong growth in selective medium. Growth was assessed by plating cells at three different dilutions.

RNA extraction and real-time RT–PCR

Total RNA was extracted using the Trizol reagent (Invitrogen), and RT–PCR was performed with the ThermoScript RT system (Invitrogen). The LightCycler system with the FastStart DNA Master SYBR Green I (Roche) was used for real-time RT–PCR. The concentration of Arabidopsis *actin* (*ACT2*) mRNA in each sample was determined to normalize for differences of total RNA amount. The data were derived from at least three independent experiments performed in duplicate. To avoid amplification of contaminating genomic DNA, primers were designed to scan for exon–exon junctions. The primer sequences used are available on request.

Purification of recombinant proteins and pull-down assays

The *TTG1* cDNA was amplified by PCR and cloned into the pGEM-T Easy vector. It was digested with BglII and religated to generate the *TTG1* clone (encoding deletion of amino acids 98–232). Both were digested and subcloned into the pGEX-KG (Pharmacia) for expression in bacteria as GST-fusion recombinant proteins. The

GEM cDNA and its deletions NtGEM (coding for amino acids 1–170) and CtGEM (171–299) were amplified by PCR and cloned in pGEM-T Easy vector. All were subcloned into the pRSET-B vector (Invitrogen) for expression in bacteria as 6His-fusion recombinant proteins. All proteins were expressed in *Escherichia coli* BL21 Rosetta after growth for 2 h at 30 °C in the presence of 0.4 mM of isopropylthio-β-galactoside, and purified using glutathione–Sepharose beads (Amersham Biosciences) or Ni-NTA agarose beads (Quiagen), as needed. For the pull-down assays, 2 μg of GST–TTG1 or GST–TTG1 bound to glutathione–Sepharose beads were incubated with equivalent quantities of the different 6His-tagged proteins in phosphate-buffered saline (PBS) for 2 h at 4 °C with agitation. The beads were washed 3 times with 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 2 more times with 50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 1 mM EDTA, 1% Triton X-100. Then, the samples were fractionated by SDS–PAGE and protein gel blot analysis was performed in standard conditions using monoclonal anti-His antibodies (Sigma).

Chromatin immunoprecipitation (ChIP)

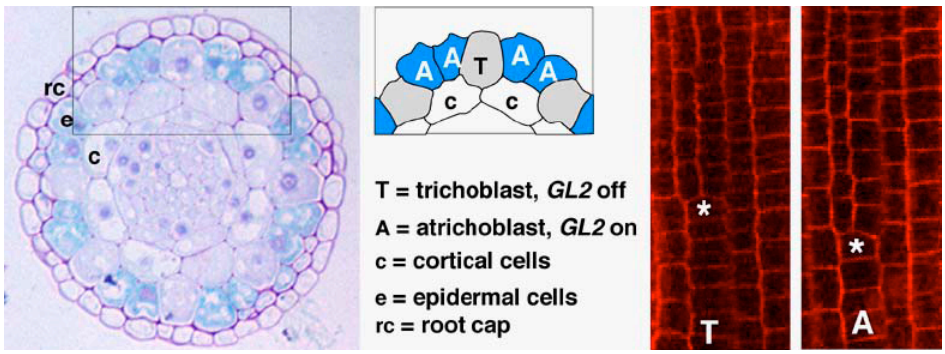
For ChIP assays 10-day-old plants were harvested and immersed in buffer A (0.4M sucrose, 10mM Tris-HCl, pH 8.0, 1mM EDTA, 1mM PMSF, 1% formaldehyde) under vacuum for 25 min. For ChIP assays 10-day-old plants were harvested and immersed in buffer A (0.4 M sucrose, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM PMSF, 1% formaldehyde) under vacuum for 25 min. Glycine was added to a final concentration of 0.1 M, and incubation continued for 10 min. Fresh material (0.3 g) was frozen in liquid nitrogen and resuspended in 1 ml of lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% deoxycholate, 0.1% SDS, 1 mM PMSF, 10 mM sodium butyrate, 1 protease inhibitor cocktail (Roche)). DNA was sheared by sonication to approximately 500–1,000-base pair fragments. After centrifugation the supernatant was precleared with 60 μl salmon sperm (ss) DNA/Protein A agarose for 30 min at 4 °C. After centrifugation, the supernatant was transferred to two Eppendorf tubes, and 10 μl of the appropriate antibody was added (anti-HA, A2095 from Sigma, and anti-acetylated H4, 06-866; anti-acetylated H3, 06-599; anti-trimethylated H3K9, 07-442; anti-dimethylated H3K9, 07-212, from Upstate Biotechnology). All antibodies, except the anti-HA, were previously bound to protein A-agarose beads. The beads were successively washed with 1 ml of 2 lysis buffer, 2 LNDET (0.25 M LiCl, 1% NP40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.0) and 3 TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). The immunocomplexes were eluted from the beads with 300 μl 1% SDS, 0.1 M NaHCO3. A total of 12 μl of 5 M NaCl was then added to each

tube, and crosslinks were reversed by incubation at 65 °C for 6 h. Residual protein was degraded by the addition of 10 μg of proteinase K (in 10 mM EDTA and 40 mM Tris-HCl, pH 8.0) at 45 °C overnight, followed by 4 phenol/chloroform/isoamyl alcohol extraction and isopropanol precipitation. Pellets were washed with 70% ethanol and resuspended in 40 μl of TE. PCR was carried out for 40 cycles. The sequences of primers used throughout this work are available on request. In the case of analysis of acetylated histones, seedlings were pretreated with sodium butyrate (10 mM) for 3 h before preparing the samples.

Synchronization of Arabidopsis cultured cells

Arabidopsis MM2d suspension cultured cells were used (Menges and Murray, 2002). Cell cycle arrest by sucrose starvation was carried out as described (Menges and Murray, 2002; Ramirez-Parra et al., 2004). Sodium butyrate (10 mM) was added to the cultures 3 h before taking each sample, to reduce histone deacetylation.

Supplementary Figures



Supplementary Figure 1: Radial organization of Arabidopsis root and epidermal cell types.
Left panel. A transverse section of the root in the meristematic region of plants expressing the GUS reporter gene (blue) under the control of the *GL2* promoter. The outermost cell layer is the root cap (rc), then the epidermis (e) and below the cortical cell layer (c). Specification of root epidermal cell fate in hair or non-hair cells is defined by a positional signal delivered by the cortical cells (c). Epidermal cells in contact with a single cortical cell are specified as atrichoblasts (A), here appearing in blue since the *GL2* gene is expressed. Epidermal cells in contact with two cortical cells take a trichoblast (T) fate, where the *GL2* gene is not expressed. For detailed reviews see ref. 1.
Right panels. In growing roots, the initial cells surrounding the columella initials originate the epidermal cell files. The occasional division of an epidermal cell in the longitudinal (anticlinal) plane originates two new cells (asterisk), one of which forms an epidermal cell clone after continued divisions in the perpendicular (anticlinal) plane. The result of these anticlinal divisions is the increase in the number of cell files that form the epidermis of the root, which will differentiate root hairs in the mature zone of the root.

a

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  1 atggagccgc cgaagggaga taccgtagtg aagactgagg tcccggtgaa ggatccatcg
 61 ctaagcgtcg tcgattcgaa aacgaaggga gtcgaggatg cgaacactga gatagccttg
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661 aaccctctct cctataaaaa cggtgatcaa accgaatgga gctattacaa ggtagtaatt
721 ccattacatc aacttaaagc agtgaatcca tcagcgagca tagtcaatcc tgcagagaag
781 tacatacagg tgatttcggt agacaatcat gagttttggt tcatgggttt tttaaactac
841 gacggcgctg tcacgtctct gcaagattct ttgcaagccg gtgctttaag gtcggtgtga

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 61 RTSSGSKKSV HWSPELVSGS QEPDQKAASS SSAGSNPYIA RSPAETSDAS LKDTMETVKG
121 VLGRWGKRVA EAAKKTESLA GNTWQHLRTA PSFADAAMGR IAQSTKVFAE GGYEKIFRQT
181 FETDPEEQLL NSFACYLSTS AGPVMGVLYI SSAKLAYCSD NPLSYKNGDQ TEWSYYKVVI
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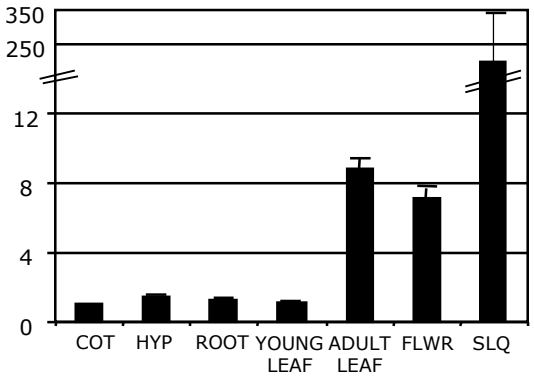
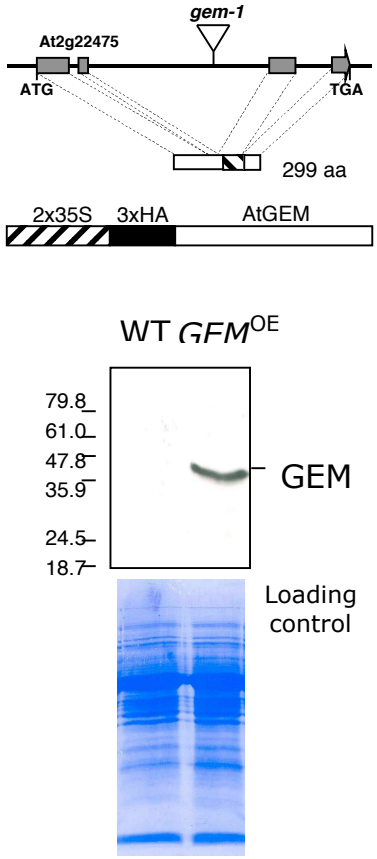
b



c

GENE		Root stage	Stele	Endo + cortex	Endo	Epidermis (A)	LRC	Peric	Colu
GL2		Stage 3	45	63	45	220		9	
		Stage 2	148	207	148	720	209	31	
		Stage 1	67	93	67	324	94		25
GEM		Stage 3	397	487	468	415		414	
		Stage 2	637	780	750	666	688	663	
		Stage 1	403	494	474	421	435		491

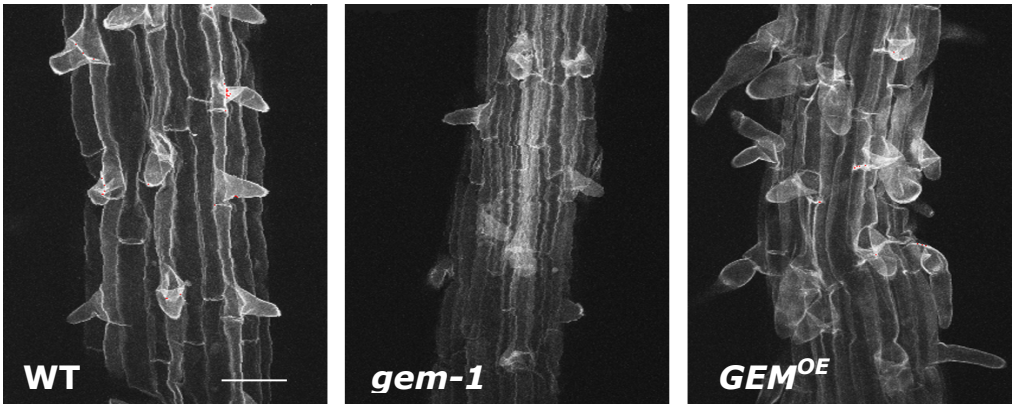
Supplementary Figure 2: Expression pattern of *GEM*.
a) cDNA and amino acid sequence of *GEM* (GenBank Acc. #EF490993). This sequence refers to At2g22475.1 at http://www.arabidopsis.org.
b) The mRNA levels of *GEM* determined by real-time RT-PCR were made relative to the amount detected in cotyledons (COT). HYP, hypocotyl; FLWR, flowers; SLQ, mature siliques. Samples for COT, HYP and ROOT were prepared from 10 day-old seedlings, for YOUNG LEAF from leaves #1/2 (14 day-old plants), and for ADULT LEAF from leaves 3/4 (34 day-old plants). Values are mean ± s.d. (n=2).
c) Expression levels of *GL2* and *GEM* genes in the Arabidopsis root in the cell types indicated at the top (Endo, endodermis; LCR, lateral root cap; Peric, pericycle; Colu, columella) depending on the location (Stages 1, 2 and 3 according to Birnbaum et al., 2003). Data were taken from http://www.arexdb.org. Note that GEM is expressed at higher levels in the upper meristem/transition zone, although it is also detectable in the other regions, in agreement with our *in situ* hybrization analysis (unpublished data).



Supplementary Figure 3: Intron-exon organization of the *GEM* locus and detection of GEM in the *GEM*^{OE} plants.

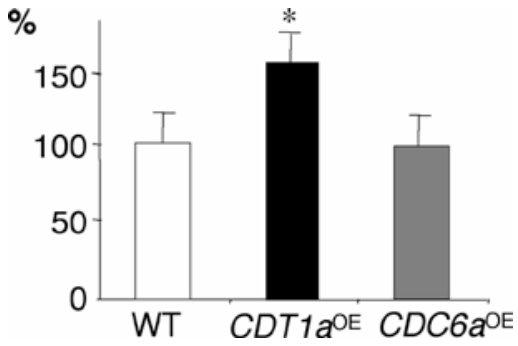
Upper panel, Intron-exon organization of the *GEM* locus (At2g22475). The position of the T-DNA insertion (*gem-1*) in the second intron is shown. The construct containing an HA-tagged *GEM* cDNA, used to generate plants with increased GEM levels (*GEM*^{OE}) is also shown.

Lower panel, GEM protein was detected in extracts of 10 day-old transgenic plants expressing an HA-tagged version using anti-HA antibodies. The mobility of the detected protein is slightly slower than the predicted molecular mass of HA-GEM protein.



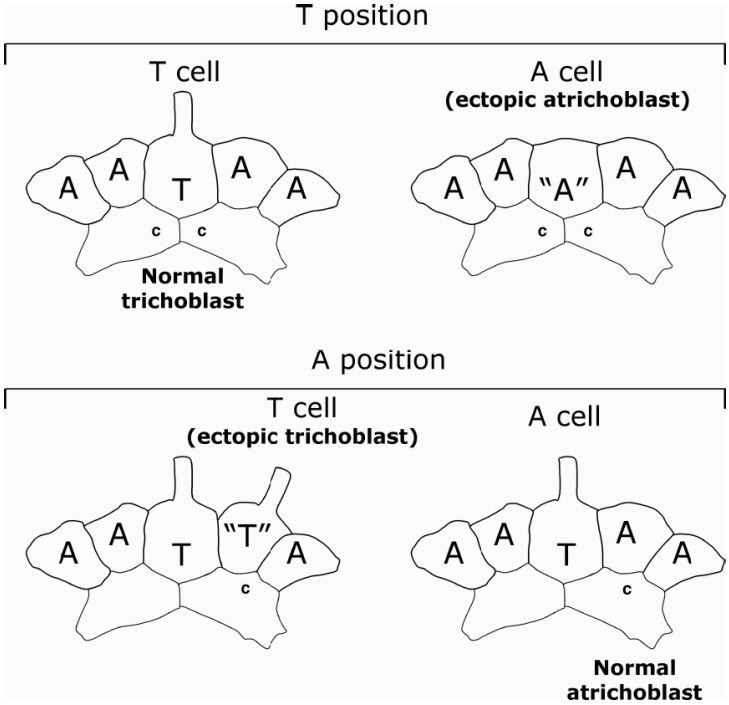
Supplementary Figure 4: Root epidermis at the beginning of the differentiation zone.

Confocal images of root the epidermis in wild type (WT), *gem-1* and *GEM*^{OE} plants at the beginning of the differentiation zone of the root (scale bar, 25 μm). Note the change in the density of developing root hairs and the loss of alternating hair/non-hair cell files as a consequence of altered levels of GEM.



Supplementary Figure 5: Effect of CDT1 and CDC6 on the frequency of longitudinal anticlinal divisions in the root epidermis.

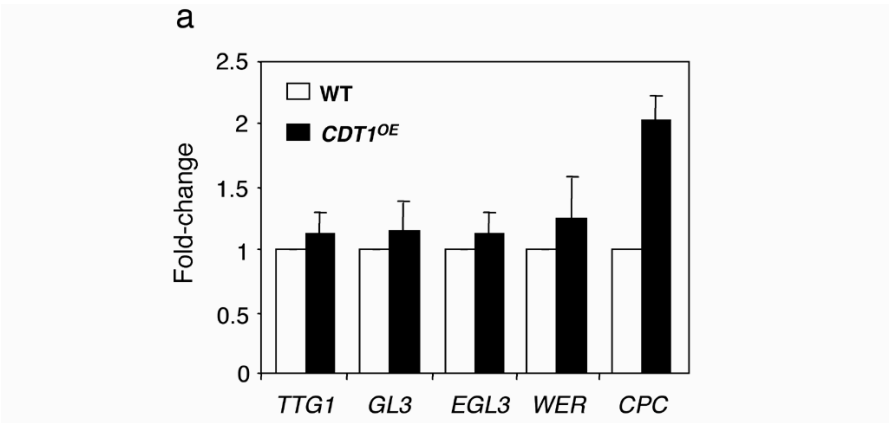
Cell walls were visualized with propidium iodide, as described in the legend to Fig. 2b. The frequency of cell clones per cell file in the indicated plants was determined. Values are mean ± s.e.m. (n = 3). Note the increase, significant though small, in the CDT1 overexpressor plants that correlates with that of *gem-1* plants. This not observed in plants overexpressing CDC6.



Supplementary Figure 6: Cell patterning in the root epidermis.

Normal specification of epidermal cells into hair and non-hair cells (see also Supplementary Fig. 1). Cells in a T position (upper panel) are normally specified as trichoblasts (T; left) since they are in contact with two cortical cells (c). Incorrect specification of these cells would produce an ectopic atrichoblast (A) in contact with two cortical cells (right).

Cells in an A position (lower panels) in contact with only one cortical cell are specified as atrichoblasts (A; right). When cell fate is incorrectly specified they differentiate into an ectopic trichoblast (left). The proportion of normal and ectopic trichoblasts and atrichoblasts was calculated by determining the amount of epidermal cells with each of the four possible patterns described above. The effect of GEM on the appearance of ectopic trichoblasts and atrichoblasts is shown in Fig. 3c.



b

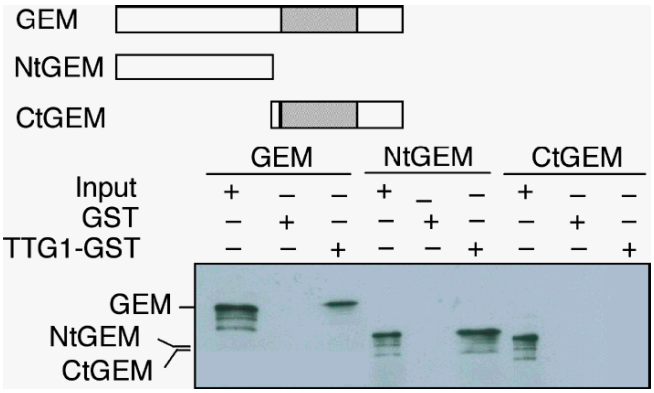
	CDT1 mRNA relative levels
wt	1
gem-1	1.06 ± 0.22
GEM^{OE}	1.25 ± 0.07

	GEM mRNA relative levels
wt	1
CDT1^{OE}	0.94 ± 0.01

Supplementary Figure 7.

a) mRNA levels of the indicated genes were determined by real-time RT-PCR, as indicated in the Methods (online), in extracts of *CDT1*^{OE} seedlings (10 day-old). Values were first normalized to the amount of *actin* gene (*ACT2*) and then made relative to the mRNA amount in wild type. Values represent the mean ± s.d. (n = 3). Note that the increase in *CPC* mRNA levels in these plants parallels that of *GL2* in the same plants (shown in Fig. 1a).

b) mRNA levels of *CDT1* and *GEM* were determined by real-time RT-PCR in extracts of *gem-1*, *GEM*^{OE} and *CDT1*^{OE} seedlings (10 day-old). Values were normalized as described for panel a, and they represent the mean ± s.d. (n = 3). Note that *CDT1* expression levels were not dependent on the levels of *GEM*. Likewise, *GEM* expression is not affected in the *CDT1*^{OE} plants.



Supplementary Figure 8: The N-terminal moiety of GEM is sufficient for interaction with TTG1.

Pull-down experiments using full-length TTG1 and truncated versions of GEM, as indicated.

Acknowledgements

We acknowledge the technical help of A. Diaz and C. Vaca. We thank J. Schiefelbein for the *pGL2-GUS*, *scm-2* and *ttg1-1* seeds, and specially Milagros Guerra (Electron Microscopy Service, CBM) for the root sections, L. Tormo for the scanning electron microscopy, and J. Schiefelbein, M. A. Blasco, B. Scheres, P. Benfey, L. Serna, E. Martinez-Salas, and J. A. Tercero for comments.

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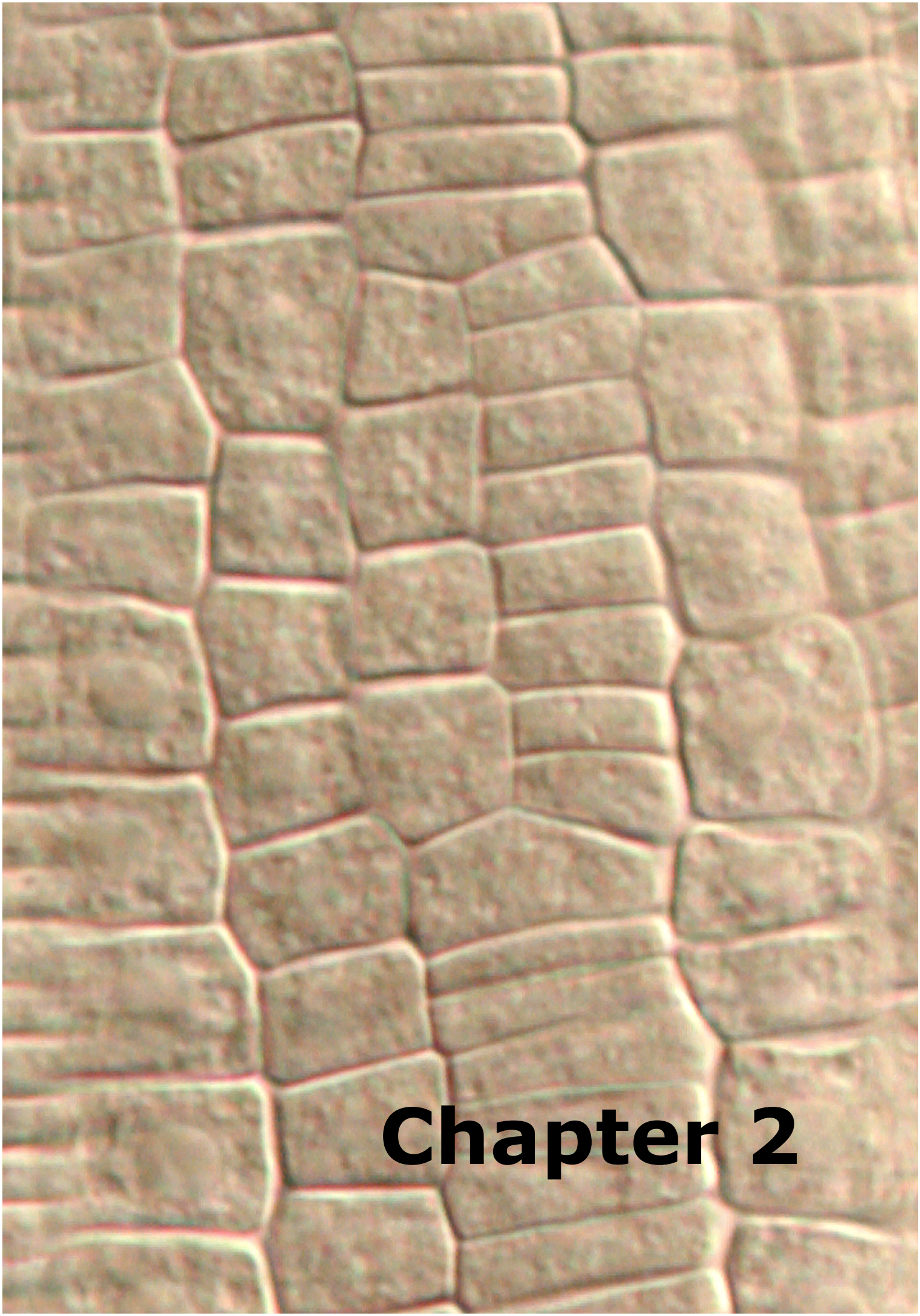
Chapter 2

A T-clone in a Normarski image of the epidermis of an Arabidopsis root meristem

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Chapter 2:

A green GEM: distinct from geminin but with intriguing analogies

A green GEM: distinct from geminin but with intriguing analogies

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Abstract

The transition of precursor cells from an undifferentiated proliferative state to differentiated cells with specific fates is of primary importance for multicellular organisms. Animals and plants have evolved two unrelated proteins, geminin and GEM, respectively, that play analogous roles in regulating this transition. These proteins are involved, probably in early G1 phase of the cell cycle, in regulating the expression of genes involved in cell fate and initiation of differentiation. They also interact with Cdt1, a component of the pre-replication complexes involved in DNA replication licensing in early G1 phase. The interaction of geminin and GEM with Cdt1 and transcriptional regulators is competitive, suggesting that these interactions can play a pivotal role in coordinating DNA replication, cell division and cell fate decisions.

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Introduction: Embryonic and post-embryonic organogenesis

Organogenesis and development of multicellular organisms depend on the generation of a pool of progenitor cells derived from totipotent and multipotent stem cells, which then undergo cell differentiation processes to give rise to the variety of specialized cells that make up an adult organism. This involves a diverse set of developmental cues, including hormonal and positional signals, integrated in both time and space. Developmental strategies in animals and plants share the need for such strict coordination; however, they are fundamentally different in several respects. Organogenesis in animals is, in general, finalized during embryogenesis, produces a constant number of organs of a fixed position and does not occur in the adult. Contrary to this, the number of organs in an adult plant body is not predetermined. Organ initiation and growth are post-embryonic processes that occur in a continuous manner during the entire lifespan of a plant, which can be hundreds of years. Stem cells in localized niches continuously provide progenitor cells that are amplified and subsequently undergo differentiation (Scheres, 2007). Another major difference between plant and animal developmental strategies is that plants can frequently regenerate new organs from differentiated tissues (Zimmerman, 1993).

All different versions of organogenesis have in common the need to arrest progenitor cell division and to acquire different cell fates before differentiation. This requires drastic changes in transcriptional networks, from controlling the production of new cells to regulating specialized cellular functions. The identification of factors and cellular conditions that control this transition is of primary importance. Here, we present a comparative evaluation of the role played by geminin in animals and by GEM (*GL2*-expression modulator) in plants. Despite being apparently unrelated, these two proteins nevertheless possess a series of intriguing analogous functions through their participation in controlling cell division, chromatin structure and the status of histone epigenetic marks of cell fate and differentiation genes. We propose that GEM, like geminin, plays a pivotal role in controlling the switch from proliferation to differentiation.

DNA replication licensing and cell fate decisions look out of the same cell cycle window

DNA replication licensing occurs in late metaphase and early G1

Eukaryotic DNA replication is achieved by a large set of proteins acting in a sequential and coordinated manner (DePamphilis, 2006). One intrinsic property of

eukaryotic DNA replication is that cells must ensure that their genome is replicated once, and only once, during each division cycle. This is largely achieved by exerting a tight control over the activation of replication origins (Blow and Dutta, 2005; DePamphilis et al., 2006).

The assembly of the pre-replication complexes (pre-RCs) is the initial step of DNA replication, a process known as DNA replication 'licensing'. Most pre-RC components have been conserved throughout eukaryotic evolution (DePamphilis, 2006). The origin recognition complex (ORC) is the first to associate with DNA at the sites where DNA replication could potentially initiate. ORC serves as a scaffold for the recruitment of the initiator protein Cdc6, rapidly followed by the incorporation of its partner, Cdt1. The Cdc6–Cdt1–chromatin complex then acts as a loading platform for the minichromosome maintenance (MCM) complex (DePamphilis et al., 2006). Unscheduled increase of the level of pre-RC components provokes abnormal triggering of DNA replication events. This may take one of two forms: massive re-replication at multiple sites in the genome, as occurs in fission yeast and human cells containing high levels of Cdc18 (the Cdc6 homologue) (Blow and Dutta, 2005) or Cdt1, respectively (Vaziri et al., 2003; Nishitani et al., 2006); or endoreplication, which consists of multiple rounds of genome duplication without mitosis, as occurs in plants (Castellano et al., 2001; Castellano et al., 2004). The licensing process relies on both the existence of low cyclin-dependant kinase (CDK) activity (regulated by the anaphase-promoting complex) and the accessibility of origin sites to the pre-RC components, which in turn depends on a favorable (i.e. 'open') chromatin state son after chromosome segregation and is maintained until early G1 (Figure 1).

The metaphase and early G1 window is also used for cell fate decisions

In spite of their different developmental strategies, both animals and plants transduce developmental cues to orchestrate complex transcriptional networks that regulate cellular transitions. These include stem cell renewal, proliferation of progenitor cells within defined transit amplifying compartments, cell cycle withdrawal and, ultimately, cell fate decisions and the initiation of specific differentiation programs.

The characteristic migration of cells and cell layers during animal embryogenesis to form new organs does not occur during plant organogenesis. Here, the presence of a rigid cell wall maintains newly formed cells joined together after cytokinesis, precluding cellular displacement. Plant organogenesis and the inherent cell fate and

differentiation decisions must be uniquely coordinated with cell division, growth and expansion (Blilou et al., 2002; Fletcher, 2002; Gutierrez, 2005; De Veylder et al., 2007; Scheres, 2007).

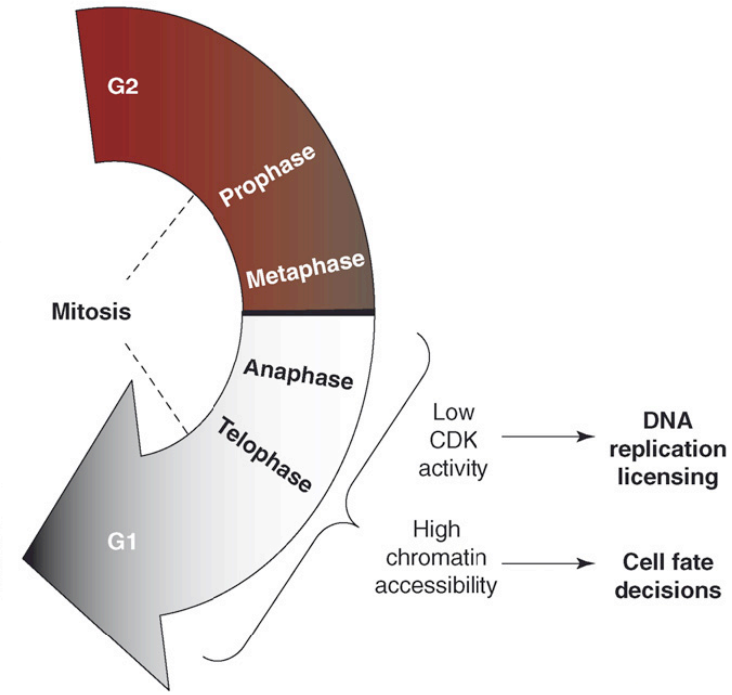
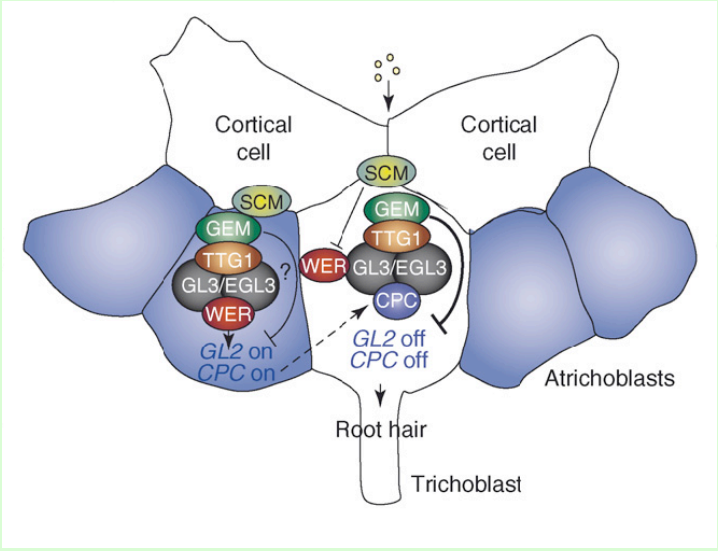


Figure 1. DNA replication licensing and cell fate specification.
The activity of the anaphase-promoting complex determines the down-regulation of CDK activity at the metaphase–anaphase transition. This allows pre-RC assembly in late mitosis and early G1. Chromatin accessibility at the *GL2* promoter is high in late M and early G1. This allows a window to interpret positional signals that determine the expression of the *GL2* gene and specifies cell fate of the newly born cells.

These cells face the challenge to respond to hormonal, developmental and environmental signals, to decide their fate. The interpretation of positional information, which also operates during animal development (Wolpert, 2003), takes a special relevance during plant development and growth. CDT1 plays a dual role in Arabidopsis by stimulating the increase both in the ploidy of cells genetically programmed to undergo endocycles and in cell division in cells with limited stem cell potential (Castellano et al., 2004), suggesting that it can regulate DNA replication licensing and cell proliferation. In addition, high levels of CDT1 increase the expression of the homeobox *GLABRA2* (*GL2*) gene (Caro et al., 2007), a key component in cell fate specification (Box 1), suggesting a connection between DNA replication components and cell fate specification.

When do plant cells decide on their fate during the cell cycle (Box 1)? This has been addressed by using 3D fluorescence in situ hybridization (FISH) to measure the

accessibility of the *GL2* locus in the developing root cells. FISH signals are detected in the anaphase nuclei and it is in the next G1 when cell fate is reassessed; the *GL2* locus remains accessible in atrichoblasts and becomes inaccessible in trichoblasts (Box 1) (Costa and Shaw, 2006). Moreover, *GL2* expression is cell cycle regulated (high in G0 and G1 and low in G2) in Arabidopsis synchronized cells (Caro et al., 2007). This pattern is due to cell cycle-dependent chromatin changes in the *GL2* promoter, which contains activating (H3ac and H3K9me3) and repressive (H3K9me2) marks in G1 and G2–M, respectively. It seems that the late M–early G1 transition is a critical time to confer accessibility, or not, to the *GL2* promoter chromatin, thus determining the 'on'/'off' state of *GL2* and the fate of the two newly formed cells. Therefore, cell fate decisions probably take place during the same cell cycle window in which DNA replication licensing occurs (Figure 1). We discuss below how these two unrelated proteins, geminin and GEM, nevertheless use analogous strategies to couple cell division, DNA replication and cell differentiation.



Box 1.
Cell fate specification in the root epidermis.

The Arabidopsis root is made up of four concentric cylinders of single-cell layers (epidermis, cortex, endodermis and pericycle) that encircle the vasculature and the inner parenquimal cells. The epidermis is the outermost cell layer and it contains two cell types. Specification of root epidermal cell fate in hair or non-hair cells is defined by a positional signal delivered by the cortical cells. Epidermal cells in contact with two cortical cells take a trichoblast cell fate (epidermal cell in white) in the proliferating part of the root, the meristem. These cells will eventually differentiate into root hair cells. Epidermal cells in contact with a single cortical cell are specified as atrichoblast (epidermal cells in blue) owing to the expression of the homeobox *GLABRA2* (*GL2*) gene. *GL2* expression in atrichoblasts is activated by a multiprotein complex containing the WD40-repeat protein TRANSPARENT TESTA GLABRA1 (TTG1), the bHLH proteins GLABRA3 (GL3) and ENHANCER OF GLABRA3 (EGL3) and the Myb transcription factor WEREWOLF (WER). This complex also activates the expression of *CAPRICE* (*CPC*), another Myb protein that moves to the neighbor atrichoblast cell. where it takes the place of WER and represses *GL2* as well as its own expression. SCRAMBLED (SCM) is a receptor-like kinase that interprets unknown positional signals in trichoblast cells. For further details the reader is directed to reviews (Berger et al., 1998; Larkin et al., 2003; Guimil and Dunand, 2006). GEM (GL2 EXPRESSION MODULATOR) represses *GL2* and *CPC* expresión by binding to the transcriptional complex through its interaction with TTG1 (Caro et al., 2007).

Geminin: dual roles in cell division and differentiation through transcriptional regulation

Geminin was first identified as a protein showing a cell cycle-dependent proteolytic pattern (McGarry and Kirschner, 1998). Independently, geminin was identified in an expression screen for proteins that affected embryonic development of the *Xenopus laevis* nervous system (Kroll et al., 1998). Geminin expression continuously increases from mid S-phase to late G2 and is very high in cells with a high proliferative rate (e.g. embryonic and cancer cells (Wohlschlegel et al., 2002)). The retinoblastoma (Rb)–E2F pathway regulates geminin expression in dividing cells (Markey et al., 2004; Yoshida and Inoue, 2004), although other transcription factors also participate (Taylor et al., 2006). The reader is directed to recent articles reviewing the role of geminin in cell proliferation and development (Luo and Kessel, 2004; Seo and Kroll, 2006; Kroll, 2007), and in DNA replication (Li and Blow, 2004; DePamphilis et al., 2006; Xouri et al., 2007a).

Geminin contains an N-terminal domain required for nuclear localization, neural cell fate functions and proteolytic degradation (Figure 2a) (Benjamin et al., 2004). Its crystal structure has revealed a central domain that contains a prototypical coiled-coil domain (Lee et al., 2004). The C-terminal domain contains a highly acidic amino acid motif implicated in the interaction with Brg1, a Brahma-related component of the SWI–SNF chromatin remodeling complex (Seo et al., 2005). Geminin sequences, which are not present in plants, have diverged considerably during animal evolution — amino acid conservation can be as low as 15% (human versus *Caenorhabditis elegans*) — although the major protein domains and its modular organization are highly conserved.

Geminin is involved in the regulation of DNA replication through its inhibitory activity on Cdt1 (Wohlschlegel et al., 2000; Tada et al., 2001). Recruitment of geminin onto chromatin by Cdt1 (Gillespie et al., 2001; Maiorano et al., 2004; Xouri et al., 2007b) inhibits MCM loading (Wohlschlegel et al., 2000). Geminin prevents re-licensing in late G2, but once geminin is destroyed at the metaphase–anaphase transition, origins can be licensed again in early G1 nuclei. Thus, high levels of Cdt1 obtained by overexpression (Vaziri et al., 2003; Thomer et al., 2004; Maiorano et al., 2005), inhibition of its proteolysis (Zhong et al., 2003; Arias and Walter, 2005; Li and Blow, 2005) or down-regulation of geminin (Quinn et al., 2001; Melixetian et al., 2004; Zhu et al., 2004; Gonzalez et al., 2006) lead to abnormal genome re-replication.

Hox and Six3, homeodomain-containing transcription factors that promote the

expression of differentiation genes, compete against Cdt1 for interaction with geminin (Figure 2b). Thus, loss of geminin leads to enlarged organs, resembling the Six3 gain-of-function phenotype, whereas cells overexpressing geminin fail to proliferate (Del Bene et al., 2004). Similar antagonistic interactions have been observed for geminin and Hox (Luo et al., 2004).

These results support a role of geminin as a cellular switch that controls gene expression, DNA replication events and cell proliferation (Figure 2b) (Seo and Kroll, 2006). Apart from regulating Hox activity by direct Gmn–Hox interaction, geminin also represses *Hox* gene expression (Luo et al., 2004). It is known that maintenance of *Hox* expression pattern depends on the balance between activator Trithorax Group (TrxG) and repressor Polycomb Group (PcG) complexes (Ringrose and Paro, 2004). Thus, geminin can have a 'PcG-like' effect by direct association with the PcG complex at *Hox* gene promoters (Figure 2c) (Luo et al., 2004). In addition, geminin interacts with the SWI–SNF chromatin remodeling complex through its Brg1 subunit, thereby blocking recruitment of this complex by basic helix–loop–helix (bHLH) transcription factors required for target gene expression (Figure 2c) (Seo et al., 2005). Finally, geminin also plays a crucial role during the transition between cell division and differentiation (Kroll, 2007), because geminin levels are high in progenitor cells and organogenesis can initiate only when geminin levels decrease by proteolysis (Figure 2d).

A green GEM: roles in cell division, cell fate and histone H3K9 modification

GEM (Box 1) was identified in a screen for CDT1-interacting proteins (Caro et al., 2007), a property it shares with geminin. However, an effect of Arabidopsis GEM on DNA replication, if any, still needs to be experimentally assessed. GEM does not contain the typical coiled-coil domain of geminin but it has a glucosyltransferase, Rab-like GTPase activators, myotubularins (GRAM) domain in its C-terminal half (Figure 2e). The role of this domain is not presently known, although it is present in proteins involved in phosphoinositol metabolism and membrane-associated processes, among other functions (Begley et al., 2003; Lorrain et al., 2004). These structural differences and the absence of GEM homologues in animals strongly suggest that geminin and GEM are unrelated proteins. Loss of the C-terminal half of GEM is sufficient to increase epidermal cell division, to change the cell type ratio in various organs and to change cell fate, indicative of a dual role of GEM in cell proliferation and cell fate specification (Caro et al., 2007). The N-terminal moiety of GEM is sufficient for its interaction with TRANSPARENT TESTA GLABRA1 (TTG1)

(Box 1), which competes with CDT1. This resembles the competition among Cdt1, Hox and Six3 for interaction with geminin (figure 2f), although TTG1 is unrelated to Hox or Six3. Chromatin immunoprecipitation experiments (Caro et al., 2007) indicate that GEM is recruited by TTG1 to repress the *GL2* promoter (Box 1). This represor activity of GEM is analogous to that of geminin on the bHLH-containing complexes at neuronal gene promoters (Figure 2g).

Global changes in histone acetylation affect the expression of *GL2* (Xu et al., 2005), the chromatin accessibility of which is linked to cell fate decisions (Costa and Shaw, 2006). GEM mediates the appearance of repressive histone marks (low histone H3 acetylation and high H3K9me2) within a proximal region of the *GL2* promoter, providing a molecular framework for *GL2*-mediated cell fate decisions (Caro et al., 2007). It is not yet known whether GEM participates in the recruitment and/or activity of chromatin remodeling complexes, as is the case for the geminin–Brg1 and geminin–PcG interactions. However, Brahma-containing SWI–SNF complexes (Farrona et al., 2004; Hurtado et al., 2006; Bezhani et al., 2007) and PcG repressor complexes (Pien and Grossniklaus, 2007) have been identified in Arabidopsis.

Contrary to geminin, the E2F pathway is not relevant for *GEM* expression, which is much higher in cells withdrawn from the cell cycle than in dividing cells (e.g. in the root, *GEM* levels increase significantly in cells leaving the meristem and entering the differentiation zone (Figure 2h) (Caro et al., 2007). This, together with the decreased cell division rate observed in *GEM* overexpressor plants, suggests that GEM is necessary for arresting the cell cycle before entering a differentiation program.

It seems that the factors and/or the cellular state required for *GL2* repression in trichoblasts are not available during the entire cell cycle. One possibility is that these factors are subjected to targeted proteolysis at the metaphase–anaphase transition. Alternative possibilities, such as inactivation by protein modification or reversion of the inhibitory epigenetic marks, are equally plausible. In any case, this is highly reminiscent of the DNA replication licensing process described above, wherein geminin prevents re-licensing in late G2 but origins, once geminin is destroyed at the metaphase–anaphase transition, can be licensed again in early G1 nuclei. Therefore, it appears that in animals and plants the same cell cycle window, in which chromatin structure allows different proteins to gain access to DNA, may be used for origin licensing and cell fate control (Figure 1).

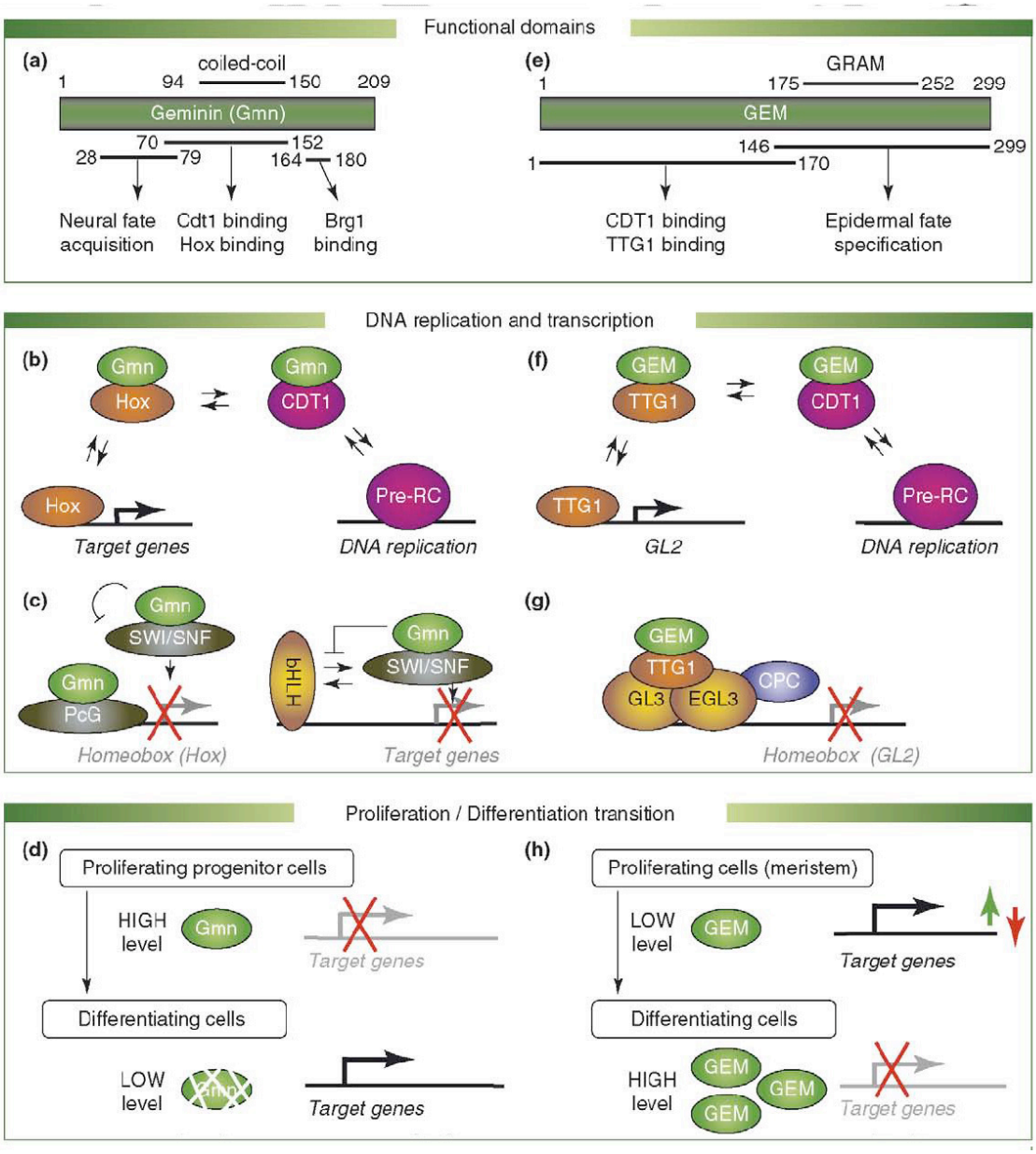


Figure 2. Comparative diagrams that summarize the structure and different roles of geminin and GEM.
(a) Structural and functional domain organization of human geminin.
(b) Competitive interaction of Gmn with Cdt1 and Hox/Six3 resulting in inhibition of DNA replication and of cell división.
(c) 'Polycomb-like' activity of Gmn and its effect on *Hox* gene expression (left) and repression of target genes by inhibiting the interaction of bHLH factors with the Brg1 subunit of activating SWI–SNF complexes (right).
(d) Dynamics of Gmn at the interface between proliferating precursor neuronal cells and the exit to differentiation. Note that precursor cells contain high levels of Gmn and that it has to be degraded before differentiation can start.
(e) Structural and functional organization of Arabidopsis GEM.
(f) Interaction of GEM with TTG1 results in repression of GL2 and other target genes. The consequences of GEM–CDT1 binding on DNA replication, if any, have not been assessed yet.
(g) Inhibition of homeobox GL2 expression by TTG1-mediated recruitment of GEM to the transcriptional complex containing the bHLH factors GL3 and EGL3.
(h) Levels of *GEM* expression in proliferating cells (e.g. root meristems) and differentiating cells. Note that *GEM* levels are relatively low in proliferating cells and much higher in cells initiating differentiation, just the opposite to Gmn.

In short, GEM and geminin appear to share several functional properties: their ability to bind Cdt1; their effect on the cell division potential; their participation in bHLH-containing transcriptional complexes; their activity as repressors of homeobox genes and genes that function at the interface between progenitor and differentiating cells; and their capacity to modulate histone marks and/or interact with chromatin remodeling complexes. In addition to their roles in proliferating cells, which still need to be fully defined for GEM, geminin seems to repress pro-differentiation genes (correlating with its high expression in proliferating cells) whereas GEM could reduce the level of repressors of such genes (correlating with its increased expression in cells withdrawn from the cell cycle), which explains the functional analogies of geminin and GEM despite their distinct expression pattern in proliferating and differentiating cellular pools.

GEM and geminin: tokens for multicellularity?

The transition from unicellular to multicellular organisms is probably one of the most radical changes that have occurred during evolution. Multicellular organization has emerged from several unicellular ancestors at independent times, both in animals and plants, but also in several lineages of fungi, amoebae and algae. Acquisition of a multicellular level of complexity was concurrent with the development of novel cellular properties derived from the need to communicate, cooperate, compete and, eventually, specialize in highly specific functions. Thus, the coordination of cell proliferation, cell fate decisions and cell differentiation is at the basis of multicellularity.

What was the molecular framework that allowed the decision of two newly formed cells to stay together, continue to divide and, eventually, to establish separate cell types with independent and coordinated functions? Appearance of novel structural and regulatory factors has been a major driving force for the unicellular to multicellular transition (Bowman et al., 2007; Ruiz-Trillo et al., 2007). Identifying these factors and their origin is a challenge necessary for understanding the emergence of multicellularity and its associated complexity (Derelle et al., 2007).

GEM homologues can be identified in higher plants (angiosperms and gymnosperms), but not in unicellular or colonial algae, diatoms (C. Bowler, personal communication), yeast and other fungi, or in animals. On the other hand, geminin homologues are present in vertebrates and invertebrates, but not in yeast and other fungi or in plants (Kroll, 2007). Animal and plant groups in which geminin

and GEM, respectively, are absent may have lost them during evolution. Also, unicellular organisms might contain proteins that share part of the functions played by geminin and GEM but have not been identified owing to low sequence similarity. However, a conceivable possibility is that GEM and geminin belong to the class of proteins associated with, or perhaps required for, multicellularity, at least in some eukaryotic lineages. In any case, it is remarkable that the functional convergence between geminin and GEM has been acquired using proteins without any apparent similarity in domain organization and in spite of the fundamental differences in organogenesis, body plan structure and developmental cues between plants and animals.

Outlook

It seems that although acquisition of GEM and geminin is associated with the transition to multicellularity, they may have appeared separately during plant and animal evolution. Although they possess an opposite expression pattern, relative to the proliferative status of the cell, they are functionally analogous in regards to their roles in cell division and transcriptional control of genes regulating the transition of proliferating cells to a differentiating state through chromatin dynamics. One intriguing analogy refers to the ability of animal geminin and plant GEM to interact with Cdt1. It would be crucial to find out whether GEM–CDT1 interaction has any inhibitory effect on the DNA replication activity of CDT1. Also, a clear effect of geminin and GEM on the proliferation potential has been documented, but the mechanisms behind it are not sufficiently understood in molecular and cellular terms. The identification of target genes whose expression is affected by these proteins, a subject that is just at its beginning in the case of GEM, is a major challenge ahead. Equally important would be to better define the functional domains of GEM and learn about its subcellular localization during the cell cycle and in different cell types.

It is conceivable that the biological processes in which GEM and geminin are involved are among the most relevant for the acquisition and maintenance of complex forms of multicellular organization. Future studies should also aim at identifying the molecular basis of the coordination among cell division, cell fate decisions and cell differentiation by these and other factors. Studies in this direction represent another example of how comparative studies in model animals and plants of basic processes of living organisms with distinct developmental strategies could be enlightening for our understanding of organogenesis in multicellular organisms.

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Chapter 3

Longitudinal view of an Arabidopsis root meristem
mounted in chloralhydrate solution

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Chapter 3:
GEM controls patterning in the Arabidopsis root meristem

GEM controls patterning in the Arabidopsis root meristem

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Abstract

Formation of an organ from progenitor cells implies two processes, cell proliferation to increase cell number, and cell fate specification and differentiation, that contribute to the generation of cell type diversity. In Arabidopsis, the root meristem contains different sets of initial cells (the equivalent of animal stem cells) located around a non-dividing set of cells, the quiescent center (QC), which maintains the initials undifferentiated state by an unknown signal. During post-embryonic root development the majority of divisions occur in the anticlinal transversal plane, originating files of cells that constitute the transit amplifying compartment or root meristem. In an occasional manner, cells also divide in the longitudinal plane contributing to the increase in the number of cell files (anticlinal division) and in the number of cell layers (periclinal division). The high frequency of anticlinal transversal divisions versus the other types suggests that mechanisms might exist that restrict the occurrence of divisions in the longitudinal plane. GEM is a CDT1 interactor protein previously involved in root development because of its role in epidermal cell fate acquisition and cell division potential control. In this work, we have analyzed GEM functions in the control of the cell division plane, potential and fate specification of other cell types within the meristem, including the stem cells.

Manuscript in preparation

Introduction

The body plan of all multicellular organisms, plants included, is complex. Formation of an organ from progenitor cells implies two main processes, namely, cell proliferation that allows the increase in cell number, and cell fate specification and further differentiation, that contributes to the generation of cell type diversity. In some cases, these two processes occur simultaneously whereas, in others, they are separated in time and space. In both situations, the spatial control of cell division and specification are crucial for patterning and proper development.

Arabidopsis roots are formed by a set of concentric cylinders, where the four outer layers, the epidermis, cortex, endodermis and pericycle surround the vascular tissue in the middle of the root. In the meristem, a non-dividing set of cells, the quiescent center (QC), control that their neighbor cells are kept in an undifferentiated state, the initial cells (the equivalent of animal stem cells). This strategy ensures that after initial cells divide, one daughter cell is kept apart from the QC and allowed to differentiate while the other one remains in the stem status (Benfey and Scheres, 2000).

During embryonic development, initials are generated by division in different spatial orientations, and then, during post embryonic development, the large majority of divisions occur in a repetitive manner in the anticlinal transversal plane, originating files of cells that together constitute the transit amplifying compartment or root meristem (Scheres, 2007). In addition, and in an occasional manner, epidermal cells divide also in the anticlinal longitudinal plane contributing to the increase in the number of cell files. Periclinal longitudinal divisions that increase the number of cell layers are extremely rare. The ground tissue initial (Benfey et al., 1993; Dolan et al., 1993) divides periclinally and longitudinally to generate separate cells for each of the two layers of ground tissue, cortex and endodermis. The low frequency of longitudinal divisions versus the transversal ones suggests that mechanisms might exist that restrict their occurrence.

Studies in mammalian cells in culture have provided insights into the molecular requirements for establishing a correct cell division plane, confirming the involvement of cell-extra cellular matrix adhesion in spindle orientation (Toyoshima and Nishida, 2007). In plants there exist important restrictions imposed by the cell wall, and the correct orientation of the separating cell walls between the two daughter cells is secured by specialized cytoskeletal structures that guide the newly formed cell plate toward a predefined cortical position. A ring of microtubules called

preprophase band defines a cortical zone that corresponds to the future division plane (Van Damme and Geelen, 2008).

Control over the division plane is crucial for root architecture, however genetic studies indicate that it is not relevant for pattern formation (Traas, 1995). Other regulatory mechanisms, relying on positional information and/or cell lineage, determine tissue organization (Willemsen and Scheres, 2004). These mechanisms are higher up in the hierarchy of the cell patterning control and are responsible for the correct organ formation (Van Damme and Geelen, 2008).

Recently, a novel protein, GEM, was identified as a coordinator of cell division and cell fate decision in the Arabidopsis root epidermis. In this work, we have analyzed GEM function in the spatial control of cell division and cell fate specification within the root meristem, as a key mechanism in the determination of organ pattern formation.

Methods

Plant material

Arabidopsis seedlings (*Col-0* ecotype) were grown in MS salts medium supplemented with 1% sucrose and 1% agar in a 16 h/8 h light/dark regime at 22 ºC. *gem-1* plants correspond to the Arabidopsis T-DNA insertion line SALK_145846 and GEM^{OE} plants express a haemagglutinin (HA)-tagged GEM protein, as described (Caro et al., 2007). $CDT1a^{OE}$ plants express a myc-his tagged version of CDT1a (Castellano et al., 2004). *pWOX5*:GFP plants (Blilou et al., 2005) and *pSCR*:GFP plants (Wysocka-Diller et al., 2000) were crossed to *gem-1* and GEM^{OE} plants and selected again for the double homozygosis.

Microscopy

For longitudinal and transversal division analysis, root meristems were mounted in chloralhydrate 80% w/v, glycerol 10% v/v. For starch granule visualization, plants were stained with lugol solution (Sigma) and cleared with chloralhydrate 80% w/v, glycerol 10% v/v. In both cases, roots were visualized with a MZ9.5 stereomicroscope (Leica) and an Axioskop2 Plus microscope (Zeiss) and the images were captured with a digital Coolsnap FX camera (Roper Scientific). For fluorescence imaging, plants were mounted in propidium iodide 50 μg/mL and visualized using a BioRad Microradiance confocal microscope. QC laser ablations were performed on a Leica SP2 inverted confocal laser scanning microscope as in van den Berg et al. (1997). Leaves were placed in a solution of lactic acid,

incubated at 100 ºC for 5 minutes and at RT overnight and mounted for light microscopy observation. Samples were observed with an Axioskop2 Plus microscope (Zeiss), and the images were processed with the ImageJ software for cell size measurement.

Results and Discussion

The meristematic cells in roots are organized in cell files. The predominant growth of a root takes place along its longitudinal axis, what requires transversal cell division. There is also some increase in the root thickness that requires longitudinal divisions, what means that cell divisions have to occur in such a way that the position of the daughter cells is turned 90° (Benfey and Scheres, 2000). There are two different types of longitudinal divisions: anticlinal (metaphase equator plane parallel to the radius of the root) and periclinal (plane parallel to the root circumference) (Oud, 1992). Longitudinal divisions are not common events compared to transversal divisions, and they are responsible for the increase in cells within a layer or for the duplication of a cell layer (Fig. 1).

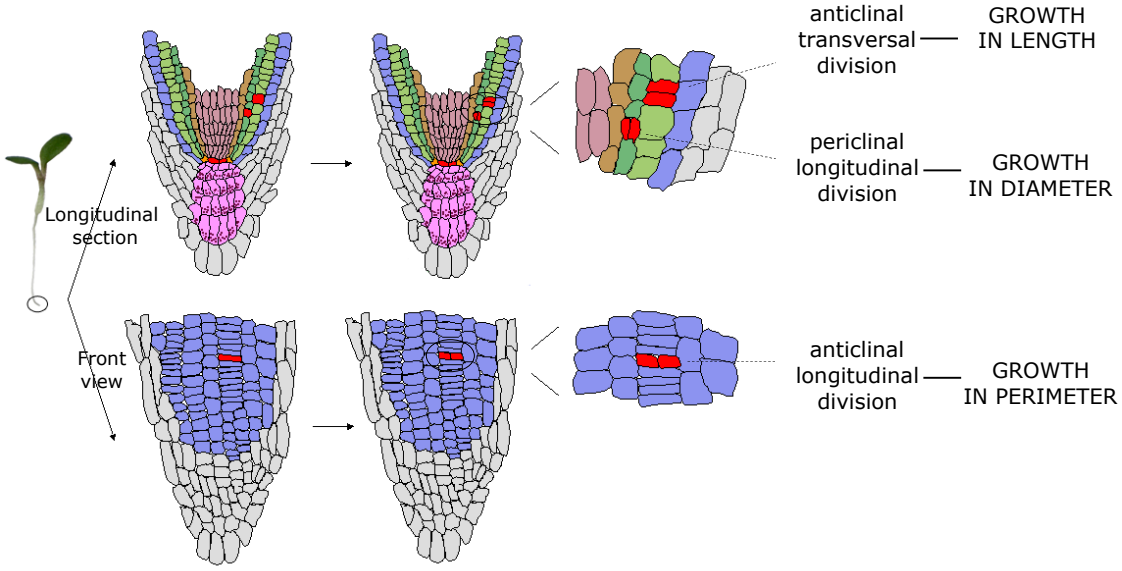

Figure 1: Schematic representation of the different types of divisions that take place in the Arabidopsis root meristem and their consequences in the formation of the root cell pattern.

In the root epidermis, GEM acts as a repressor of anticlinal longitudinal divisions that account for the increase in the number of cell files in each layer (Caro et al., 2007). To determine whether GEM also plays a role in restricting division potential in the other planes, we first assessed its effect on the size of the root division zone. We defined the meristematic region as that in which cells are actively dividing and relatively small (<13 μm in length). Once cells exit the cell cycle and start to

elongate as part of their differentiation program, they become larger (>13 μm; Fig. 2a).

The analysis showed that cell size in the meristematic region was not affected by GEM (Fig. 2b), although the location of the boundary defining where cells exit the meristem and enter the elongation/differentiation zone was altered. In *gem-1* mutants, this boundary was shifted upward in the root, as a consequence of an increase in the meristematic cell number, whereas the opposite occurred in the GEM^{OE} (Fig. 2c).

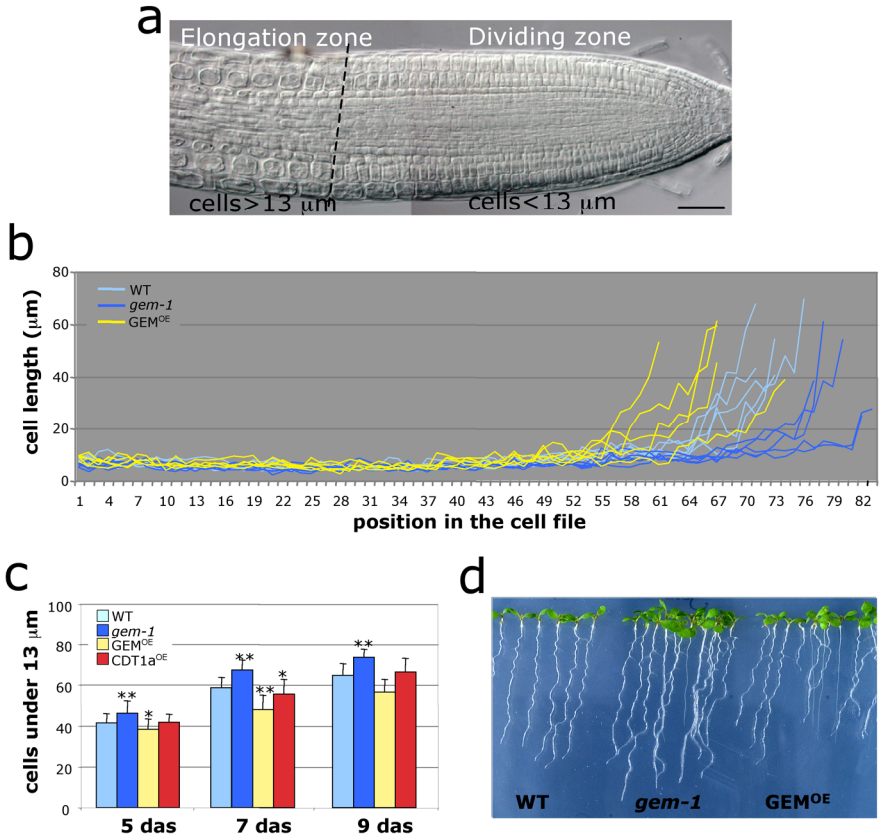

Figure 2: GEM controls meristem size
a) Scheme of dividing/elongation zones in Arabidopsis roots and criteria used to define the region referred as "meristem" (cells <13 μm in length). Scale bar, 50 μm.
b) Cortex cell length in 5 different representative WT, *gem-1* and GEM^{OE} roots. In the X-axis, position 1 represents ground tissue initial and continues with next cells in cortex cell file.
c) Meristem size at different time points in WT, *gem-1*, GEM^{OE} and CDT1a^{OE} roots. (n>10; *P<0.1; **P<0.05).
d) WT, *gem-1* and GEM^{OE} 6 das seedlings. Note the differences in root length.

CDT1 is a DNA replication protein that interacts with GEM *in vitro* (Caro et al., 2007) and *in vivo* (this Thesis, Chapter 4) and inhibits the occurrence of anticlinal longitudinal divisions (Caro et al., 2007). Interestingly, increased levels of CDT1a do not increase mersitem size (Fig. 2c). This lack of effect of CDT1a on meristem size is maintained at different times after sowing (Fig. 2c) whereas the

consequences of GEM disfunction, very striking at 9 das, were already detected early after sowing (Fig. 2c). Therefore, root length was dependent on GEM levels (Fig. 2d) and independent on CDT1a levels (data not shown). A striking conclusion risen from these observations is that control over the transversal divisions in the root meristem relies on mechanisms distinctly affected by GEM and CDT1.

Cross sections of root meristems of *gem-1* and GEM^{OE} plants showed the existence of an aberrant radial organization in the root (Caro et al., 2007), suggesting an altered pattern in anticlinal longitudinal divisions. Detailed inspection of *gem-1* plants revealed the frequent occurrence of an excess of periclinal longitudinal divisions leading to an increase in the number of cell layers (Fig. 3a-c). Although some occasional divisions could be observed in the cell layers in the position of the cortex (Fig. 3a) and the pro-vascular tissue (Fig. 3b), periclinal longitudinal divisions occurred preferentially in the layer in the endodermis position (Fig. 3c). Quantification of the frequency of periclinal longitudinal divisions in *gem-1* and GEM^{OE} plants showed that GEM acts as a repressor also for this type of divisions (Fig. 3c). Interestingly, CDT1^{OE} plants also show increased frequency of periclinal longitudinal divisions in the endodermis. This suggests that, contrary to the control of transversal divisions in the meristems, GEM and CDT1 share, at least in part, the pathway controlling the switch in cell division plane.

The function of GEM on the spatial control of cell division may suggest an indiscriminate effect as a cell division inhibitor. However, loss of GEM had a stronger effect on anticlinal and periclinal longitudinal divisions than on transversal divisions (meristem size), as shown by the fact that in *gem-1* mutants, periclinal and anticlinal longitudinal divisions occurrence show a two-fold increase in both cases (Caro et al., 2007), while transversal divisions are increased only ~ 12%. A possibility is that transversal division constitutes the default pathway for cell division in the root, whereas longitudinal divisions behave as active processes controlled by unknown mechanisms.

The cortex and the endodermis form the ground tissue. The radial pattern of the ground tissue is established early in the meristem, and SHORTROOT (SHR) and SCARECROW (SCR) transcription factors (Pysh et al., 1999) play a central role in the patterning process (Nakajima and Benfey, 2002). Later, as the Arabidopsis root ages, a third layer of ground tissue forms, which rapidly takes on cortex character (Paquette and Benfey, 2005). In the epidermis, GEM is involved in coordinating cell division and hair/non-hair cell fate acquisition.

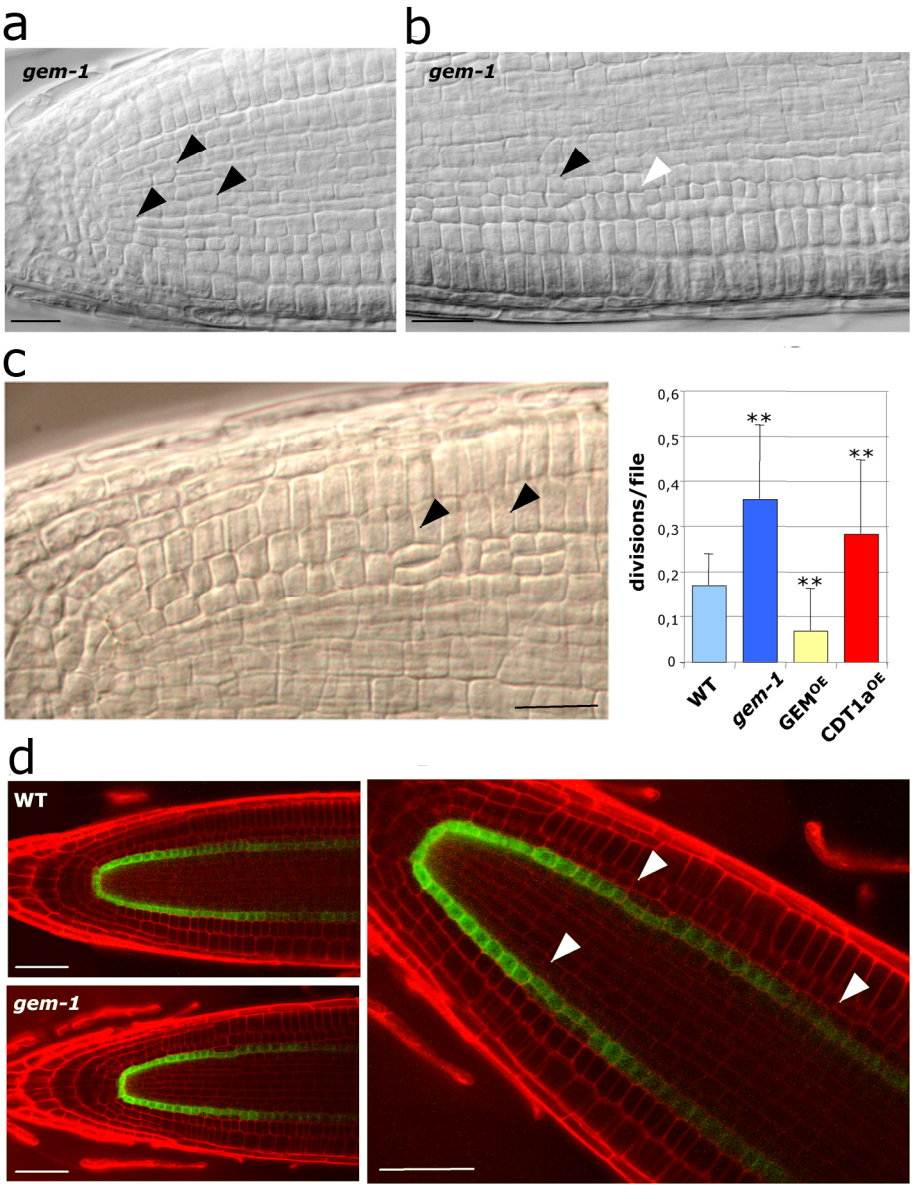

Figure 3: GEM restricts periclinal longitudinal divisions in the root meristem.
a) Periclinal longitudinal divisions in the pro-vascular tissue of 7 das *gem-1* plants (black arrowheads). Scale bar, 25 μm.
b) Periclinal longitudinal divisions in the cell layer in cortex position (black arrowhead) and endodermis position (white arrowhead) of *gem-1* root meristems of 7 das plants. Scale bar, 25 μm.
c) Left panel, periclinal longitudinal divisions in the endodermis position of 7 das *gem-1* plant meristem. Scale bar, 25 μm. Right panel, quantification of the occurrence of periclinal longitudinal divisions in WT, *gem-1*, GEM^{OE} and $CDT1a^{OE}$ root meristems in 9 das plants (n>25 roots; **P<0.05).
d) *pSCR*::GFP expression in WT and *gem-1* root meristems of 9 das plants (left panels). In the right, a detailed view of a *gem-1* root meristem where 3 longitudinal periclinal divisions can be appreciated (white arrowheads). Scale bars, 50 μm.

To investigate whether GEM also plays a role in controlling cell fate acquisition in ground tissue cells, we assessed the expression of the endodermis cell type marker *pSRC*::GFP in the different *GEM* backgrounds. This study showed that the periclinal longitudinal divisions that occur in these plants account for the normal formation of a third ground tissue layer with cortex fate, since only the inner layer attains the endodermis fate (Fig. 3d). These results indicate that endodermis/cortical fate is

not dependent on GEM, since after endodermis periclinal longitudinal cell division, only one endodermis cell layer is specified. Thus, GEM effect appears to be limited to specifying fate within the epidermal layer, which consists of two cell types, but not between different cell layers. Giberellins (GA) and SCR have been shown to additively regulate the timing of formation of the new ground tissue layer (Paquette and Benfey, 2005). Whether the effect observed in GEM mutants is only the consequence of a stimulation/repression of cell division potential or whether GEM is involved in GA signalling still needs to be studied in depth.

Organogenesis in multicellular organisms depends on the coordinated function of stem cells and their descendants, which are amplified before undergoing differentiation. Both cell differentiation and stem cell maintenance must occur at the root meristem in order to ensure the proper root growth. In Arabidopsis roots, the columella stem cells consist of a single layer immediately distal to the QC that divide generating a stem cell daughter cell and another daughter cell that will differentiate, elongate and accumulate starch granules. In *gem-1* seedlings, additional undifferentiated cells appear in the columella stem cell area, while in GEM^{OE} seedlings a general destructuration of the QC and columella zone can be found (Fig. 4a-c). To better undestand this phenotype we used the lugol staining of the starch granules to identify the differentiated columella cells. We could see that small and undifferentiated cells accumulated between the QC and the columella in *gem-1* plants, while in GEM^{OE} cells start to accumulate starch and differentiate in the position where columella stems should form a single layer (Fig. 4d-f). This suggests that GEM controls the proliferation/differentiation balance of columella stem cells.

The QC domain also appeared altered in *GEM* mutants. To determine whether the QC is still functional in *gem-1* roots and if the small and accumulated cells we find beneath the QC are stem cells, we performed QC ablation assays, which produce columella stem cell differentiation in WT roots (van den Berg et al., 1997). Ablation of QC cells in *gem-1* roots (Fig. 4g, h) induced differentiation of the undifferentiated columella cells within only 24 hours (Fig. 4i). Thus, we conclude that the *gem-1* seedlings possess a functional QC and that in these plants, extra cells have a columella stem cell nature. The occurrence of extra divisions can be noted even in the differentiated columella cells, where the typical straight pattern of the WT is lost (Fig. 4m).

The root meristem stem cell niche is constituted together, by the QC cells and the surrounding initial cells (Xie and Spradling, 2000; Xu and Scheres, 2005). Although

loss of GEM does not appear to affect QC function on columella stem cells, the QC did not have a normal appearance in *GEM* mutant plants.

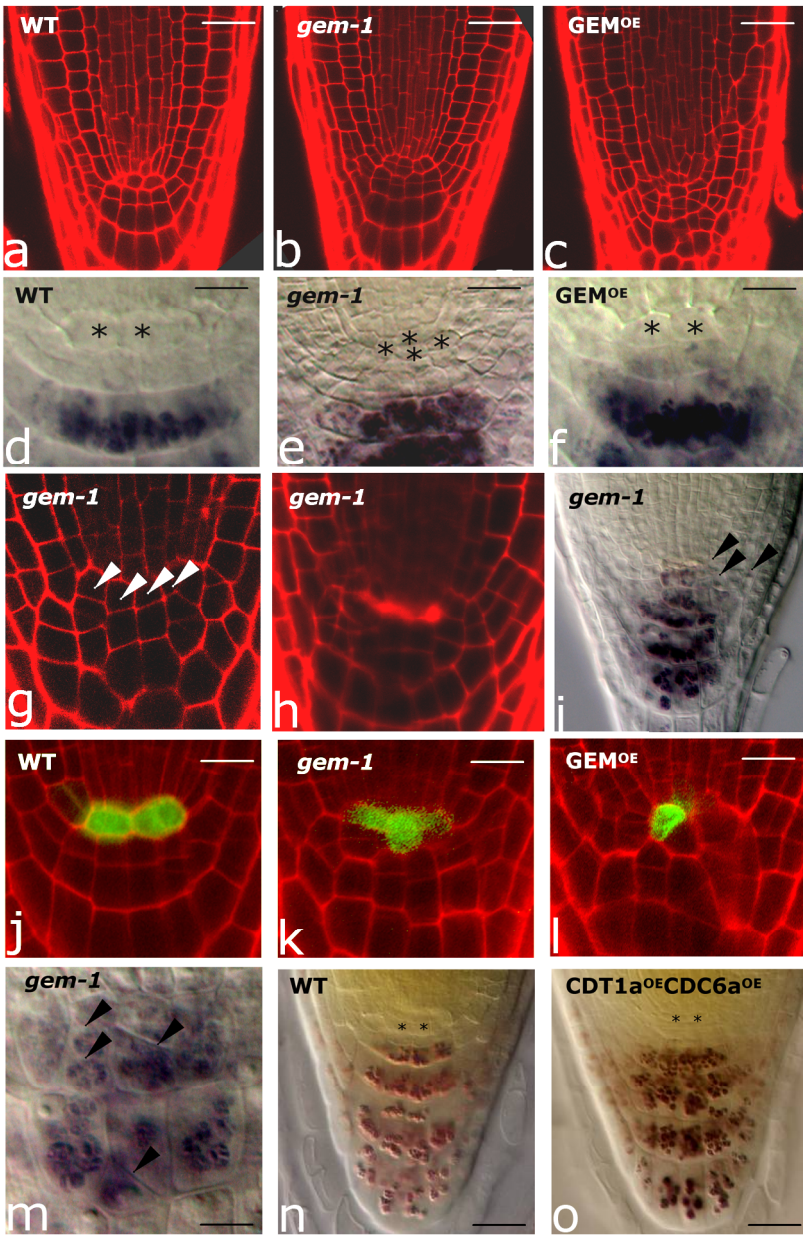

Figure 4: Columella stem cell phenotype analysis
a-c) Longitudinal confocal sections of WT (a), *gem-1* (b) and GEM^{OE} (c) root meristem QC area. Scale bar, 20 μm.
d-f) Lugol starch granule staining of differentiated columella cells in WT (d), *gem-1* (e) and GEM^{OE} (f) seedlings. Asterisks indicate QC cells position. Scale bar, 10 μm.
g) QC area of a 10 das *gem-1* seedling before ablation. White arrowheads point to the cells to be ablated. Scale bar, 10 μm.
h) Longitudinal confocal section of the root 24 hours right after ablation. Scale bar, 20 μm.
i) Lugol staining of the root 24 hours after laser ablation. Black arrowheads point to the newly differentiated cells that have started to accumulate starch. Scale bar, 10 μm.
j-l) WOX5:GFP expression analysis in WT (j), *gem-1* (k) and GEM^{OE} (l) 5 das roots. Scale bar, 10 μm.
m) Detail of abnormally divided differentiated columella cells in *gem-1* root meristem. Scale bar, 10 μm.
n, o) Lugol starch granule staining of differentiated columella cells in WT (n) and CDT1a^{OE}CDC6a^{OE} (o) 7 das seedlings. Asterisks indicate QC cells position. Scale bar, 20 μm.

To investigate the possible role of GEM on QC cell division potential we identified functionally QC cells by expressing the *WOX5* QC marker (Blilou et al., 2005) in *gem-1* and GEM^{OE} backgrounds (Fig. 4j-l). We found that in *gem-1* roots, the QC appeared often divided and *WOX5* expression shows that more than two cells maintain QC fate (Fig. 4k). On the contrary, in GEM^{OE} plants with a strong phenotype differentiation in the stem cell niche occurred, and a significant reduction in the number of QC cells was produced (Fig. 4l). These data implies that GEM acts as a general repressor of cell division in the Arabidopsis root meristem, also involved in cell division repression in columella stem cells and QC cells, keeping the division/differentiation balance necessary for assuring meristem function.

One possibility is that the mechanism of action of GEM on QC and stem cell division in the root meristem is analogous to its role in cell division and fate specification in the epidermis. This involves interaction with the cell division protein CDT1a and changes in the histone modification pattern. Excess of CDT1 does not have any detectable effect on meristem size and show normal QC and stem cell niche (Fig. 2c, data not shown). However, overexpression of both CDT1 and CDC6, the CDT1 partner, produced supernumerary columella stem cells (Fig. 4n,o), a phenotype similar to that of *gem-1* plants. The E2F/RBR pathway has already been implicated in stem cell proliferation in mammals (Liu et al., 2004) and plants (Wildwater et al., 2005). E2F-independent effects of RBR on stem cell function cannot be discarded.

However, some E2F play a clear role as stem cell promoting factors in the root (Wildwater et al., 2005). Whether CDT1 and CDC6, which are E2F targets (Castellano et al., 2001; Castellano et al., 2004), are part of the gene network controlling stem cell function in the root, remains an attractive possibility.

GEM had previously been involved in controlling cell fate acquisition in root and in leaf epidermis (Caro et al., 2007). Thus we asked whether its extensive role as a cell division repressor was specific of root development. We analyzed *gem-1* and GEM^{OE} leaves and found that cell size is slightly altered in *GEM* mutants, while organ size remains constant, what could suggest an effect of GEM as a repressor of cell division and a promotor of cell differentiation. However, the effect is very small, maybe due to the compensatory mechanisms that operate during leaf development and that can hide mild phenotypes (Fig. 5a and b).

The analysis of the stomatal complexes distribution in leaves of *GEM* mutants

showed no difference with the WT, uncovering new differences in the response of specific cell types to the same factor (Fig. 5c).

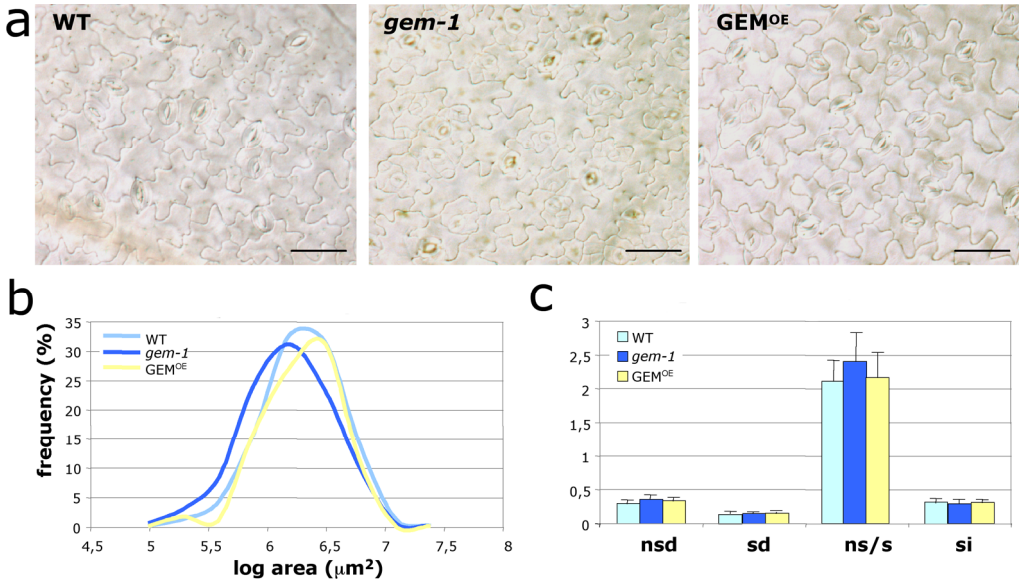

Figure 5: Cell division analysis in WT, *gem-1* and GEM^{OE} 22 das leaves.
a) Adaxial epidermis of leaves 1 and 2 from 22 das plants. Scale bar, 50 μm.
b) Epidermal cell area analysis
c) Stomatal related indexes: Nonstomatal cell density (nsd; number of nonstomatal cells per mm^2), stomatal density (sd; number of stomata per mm^2), nonstomatal epidermal cells/stomata ratio (ns/s), and stomatal index [si; (sd/sd + epidermal cell density)*100].

We can conclude that GEM acts as a general repressor of cell division in the Arabidopsis root meristem. It is controlling transversal cell division and thus, meristem size, and specifically inhibiting the change in the division plane necessary to generate the longitudinal divisions responsible for the increase of growth of the root, proving an important factor for organ pattern formation. Nevertheless, its role in leaf development, remains to be determined. Moreover, GEM is involved in cell division repression of columella stem cells and QC cells, controlling the division/differentiation balance necessary for assuring meristem plasticity. GEM implications in cell fate acquisition seem to be specific of hair/non-hair identity in root and leave epidermis, and not necessary for specification of cortex or endodermis identity in the root.

All this data confirms GEM's role in assuring a correct organ pattern and development and future studies should aim to solving the molecular mechanisms by which it acts.

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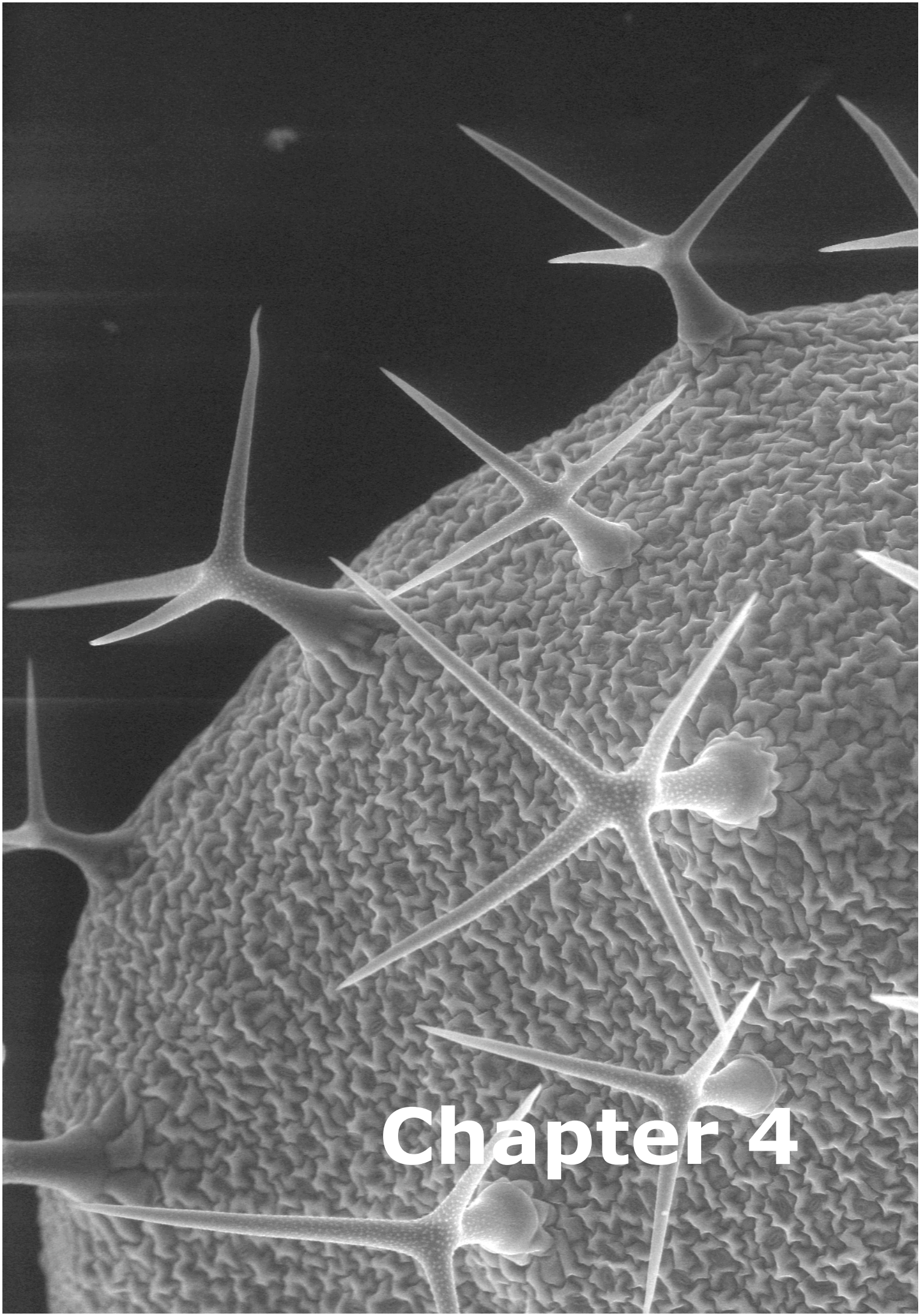

Chapter 4

Scanning electron microscopy photograph of
trichomes in an Arabidopsis leaf adaxial surface

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Chapter 4:
CDT1 is a key factor in Arabidopsis DNA endoreplication control regulated by redundant mechanisms

CDT1 is a key factor in Arabidopsis DNA endoreplication control regulated by redundant mechanisms

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Abstract

Cdt1 is a licensing factor for DNA replication, conserved among all eukaryotes and whose function is tightly controlled to maintain genome integrity. Eukaryotic organisms have evolved a wide variety of mechanisms to control the availability and function of Cdt1, including E2F control of expression, phosphorylation by Cdks, degradation and geminin binding. In this work we study the mechanisms for CDT1 control and its involvement in endoreplication in *Arabidopsis thaliana*, an organism that has proved highly tolerant to alterations in cell cycle regulators and that does not turn to transformation or oncogenic pathways as animal cells do. Here, we show that *At*CDT1 is subjected to proteasome degradation by SCF^{Skp2} similarly to what happens in human cells. The ancient degradation pathway present from yeast to mammals that involves ubiquitination by a Cul4-based complex is also conserved in Arabidopsis, though it seems to be independent from CDT1-PCNA binding. The CDT1 inhibition mechanism present in metazoans, however, does not seem to happen in plant cells, at least as a replication control pathway, but other redundant strategies might exist that still remain unknown, since an abrogation of the degradation systems is not enough to produce an over-replication phenotype in developing Arabidopsis plants.

Manuscript in preparation

Introduction

In eukaryotic cells DNA replication initiates at multiple replication origins scattered all over the genome of the cell. Activation of DNA replication depends on the formation of pre-RCs that consists of a highly ordered assembly of ORC, Cdc6, Cdt1 and Mcms. To prevent the genomic instability that re-replication would cause, the establishment of the pre-RCs at the origins of replication needs to be suppressed during S, G2 and M phases of the cell cycle. It has been demonstrated that pre-RCs regulation during the cell cycle is extremely diverse among eukaryotes, in spite of a high conservation of the proteins involved. However, in most eukaryotic organisms, Cdt1 is a key target over which the main control pathways are established (reviewed in Fujita, 2006). This suggests that Cdt1 deregulation may induce more deleterious effects than other pre-RC components such as Orc1 or Cdc6. Indeed, it has been reported that in human cells, Cdt1 overexpression can induce re-replication (Vaziri et al., 2003), whereas Orc1 or Cdc6 overexpression has no or only marginal effect on cell cycle progression (Jiang et al., 1999; Petersen et al., 1999; Pelizon et al., 2000; Vaziri et al., 2003; Tatsumi et al., 2006).

Several mechanisms have evolved to regulate Cdt1 function, including changes in the subcellular localization, modulation of DNA binding activity, degradation and direct inhibition. Cdks play a major role controlling Cdt1 phosphorylation and assuring that pre-RCs assemble during the low Cdk activity period between late mitosis and early G1 (Bell and Dutta, 2002; Blow and Dutta, 2005). However, the consequences of Cdt1 phosphorylation by Cdks are diverse depending on the system: it leads to exportation out of the nucleus in budding yeast (Tanaka and Diffley, 2002), whereas in mammalian cells it inhibits Cdt1 DNA binding (Sugimoto et al., 2004). In human cells, Cdk phosphorylation of Cdt1 on the cyclin-binding motif (Cy motif) stimulates binding to the F-box protein Skp2 (Liu et al., 2004; Sugimoto et al., 2004) and its subsequent degradation by the proteasome (Li et al., 2003; Takeda et al., 2005). An alternative mechanism that triggers Cdt1 degradation in response to UV irradiation has been demonstrated in fission yeast, Drosophila and human cells (Arias and Walter, 2006; Higa et al., 2006; Hu and Xiong, 2006; Nishitani et al., 2006; Senga et al., 2006). It is mediated by Cul4-Ddb1 complex and requires the interaction of the trimeric ring PCNA with the PCNA interacting protein (PIP) box motif of Cdt1 (Kim and Kipreos, 2007).

The last mchanism to control Cdt1 function that occurs in metazoans is inhibition by geminin. Geminin was originally identified in Xenopus and found to inhibit pre-RCs formation by preventing Mcm loading (McGarry and Kirschner, 1998). Subsequent

work has shown that geminin inhibits licensing by binding to and inhibiting Cdt1 (Wohlschlegel et al., 2000; Tada et al., 2001). Since transcription of geminin is driven by the E2F transcription factor (Yoshida and Inoue, 2004) and geminin is an APC target, the protein appears after cells enter S phase and is destroyed during exit from mitosis to allow pre-RCs formation (McGarry and Kirschner, 1998; Wohlschlegel et al., 2000; Tada et al., 2001). Based on sequence homology, a geminin homologue does not seem to be present in plants, although a CDT1-interacting protein called GEM (Caro et al., 2007) unrelated to it, exhibits interesting functional analogies to geminin (Caro and Gutierrez, 2007). However, its possible role in controlling DNA replication initiation and its impact on over-replication control has not been addressed yet.

Studies on regulation of DNA replication in multicellular organisms are scarce although they all suggest that the control of DNA licensing is important for the developmental programs to be established correctly during embryogenesis and development. Strong mutations in Dup (the Drosophila Cdt1 homologue) cause embryonic lethality (Whittaker et al., 2000). Stabilization of *C. elegans* Cdt1 causes massive DNA re-replication and lethality among the progeny (Zhong et al., 2003). Lack of geminin results in preimplantation mortality of mouse embryos, since DNA replication occurs but mitosis is not detected (Hara et al., 2006). In medaka, loss of geminin promotes retinal precursor-cell proliferation and results in expanded optic vesicles (Del Bene et al., 2004). Endoreplication, as a form of over-replication is widespread in protists, plants, and many animals including arthropods, mollusks, and mammals as part of their normal developmental programs (Porter, 2008). Overexpression of Arabidopsis CDT1 has no lethal phenotype, but leads to cell type-specific effects in developing plants: in leaf cells competent to divide, cell proliferation is stimulated, whereas in cells programmed to undergo differentiation-associated endoreduplication rounds, extra endocycles are triggered (Castellano et al., 2004).

In this work we have identified functional domains of Arabidopsis CDT1 involved in controlling its availability. We have also demonstrated that CDT1 is a target for preventing endoreplication and reinforced the idea that in plants, the switch from the cell cycle to the endocycle program may provide a safeguard mechanism in case of altered levels and/or function of CDT1. Finally, the CDT1-interacting protein GEM seems to have a limited effect, if any, on restricting the occurrence of endocycles.

Methods

Plant materials

Three independent CDT1^{OE} lines have been used in this study, (1) pROK-CDT1^{OE} line 1/4 (Castellano et al., 2004) that constitutively express CDT1a fused to a myc-his tag; (2) pOEX2-CDT1^{OE}, that constitutively express CDT1 fused to a 3xFLAG tag and (3) pTACDT1 plants that express AtCDT1a fused to a 3xFLAG tag upon dexamethasone induction. *cul4* and *skp2a*, *skp2b* mutants have been described previously (Bernhardt et al., 2006; del Pozo et al., 2006).

Recombinant protein purification and in vitro pull-down assays

The *CDC6a* cDNA was amplified by PCR and cloned into the pDONR vector. An LR reaction was performed to obtain the *CDC6a* cDNA subcloned in the pDEST17 vector (Invitrogen). The *GEM* cDNA was amplified by PCR, cloned in pGEM-T Easy vector and subcloned into the pRSET-B vector (Invitrogen) as described (Caro et al., 2007). CDT1 cDNA and its deletions (ΔNtCDT1a includes aminoacids 113-571 and ΔCtCDT1a includes aminoacids 1-475) were cloned in pGEX-KG vector for production of GST fused proteins. All proteins were expressed in *Escherichia coli* BL21 Rosetta after growth for 1 h 30 minutes at 30 °C in the presence of 0.4 mM of isopropylthio-β-galactoside, and purified using Ni-NTA agarose beads (Quiagen). For the pull-down assays, 2 μg of GST–CDT1a, or its deleted forms bound to glutathione–Sepharose beads were incubated with an equivalent quantity of the 6His-CDC6a protein in phosphate-buffered saline (PBS) for 2 h at 4 °C with agitation. The beads were washed 3 times with 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 2 more times with 50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 1 mM EDTA, 1% Triton X-100. Then, the samples were fractionated by SDS–PAGE and protein gel blot analysis was performed in standard conditions using monoclonal anti-His antibodies (Sigma).

Electrophoretic mobility shift assays

The protein-probe binding was performed in 50 mM Tris-HCl, pH 7.5, 5% glycerol, 100 mM KCl, 1 mM DTT, 0.05 % Triton X100, 0.5 mg/ml BSA, where recombinant purified GST-AtCDT1a protein and its N terminal and C terminal truncated versions were added, as indicated. Binding reactions were incubated for 20 min at room temperature, and the DNA–protein complexes were fractionated by electrophoresis in 4% polyacrylamide gels in 0.5% Tris-borate/EDTA (TBE) buffer. Synthetic oligonucleotides of 20 and 52 nucleotides (5'-AGATAGCCTTGTCCGATGAG-3' and 5'-CTAACAAAAATGCAGAGAAAGAGAAAGAGATTAAGAGAGTATTGATACATGA-3') were labeled with γ-^{32}P-ATP and used as binding substrates. In the cases "ds" is indicated, oligos were annealed with an excess of the cold complementary bottom

strand to generate double stranded DNA.

In vivo pull down assays

For PCNA/Cdt1 interaction assays, Arabidopsis Col0 plants were grown for 7 days in liquid MS medium (Duchefa). Plants were harvested, washed, grinded in liquid nitrogen and resuspended in 50 mM Tris-Hcl, pH 7.5, 50 mM NaCl, 0.2 % NP40, 1mM PMSF and protease inhibitor cocktail (Sigma) to produce an extract of 1mg/ml protein concentration. Then, 1μg of GST-fused recombinant protein was added and incubated with 1 ml of extract for about 2 hours at 4ºC. Three washes were performed, the two first ones with the same extraction buffer and the last one with 100 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5 % NP40. The bound proteins were eluted, fractionated by SDS-PAGE and subjected to Western Blot analysis with anti-PCNA mouse monoclonal antibody. For SKP2-CDT1 interaction assays, the same protocol was used, but the extracts were produced from plants that constitutively express a myc tagged version of AtSKP2a (del Pozo et al., 2006). Extracts were incubated with 5mM ATP, 5mM Creatine Phosphate, 10 U/ml Creatine kinase and 0.05 μg/ml ubiquitin for 5 minutes prior adding recombinant proteins when "phosporylating conditions" is indicated. The extracts were incubated with roscovitine (50 μM) for 30 minutes prior to recombinant protein addition, as indicated.

Yeast two hybrid assay

For the yeast two-hybrid assay, yeast cells (HF7c strain) were transformed with the corresponding constructs, pGBT8-CDT1a, pGBT8-ΔNtCDT1a or pGBT8-ΔCtCDT1a and pGAD-GEM, pGAD-ΔNtGEM, pGAD-ΔCtGEM (truncated versions described in Caro et al., 2007) or pGAD-ΔGRAMGEM (that includes aminoacids 1-174 plus 253-299). Cells were grown for ~3 days, and the co-transformants selected in minimal synthetic defined (SD) media plus histidine. Interaction assays were performed in SD media.

Immunoprecipitation

Anti-myc 9E10 epitope monoclonal antibodies (Santa Cruz) were conjugated for 1 h with Protein A-agarose at room temperature in PBS 1x. AtCDT1a-myc overexpressor plants were grinded in liquid nitrogen and resuspended in 50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.2% NP40, 1mM PMSF and protease inhibitor cocktail (Roche). ProteinA-agarose antibodies were added to the extracts and the mixture was incubated for 12 h at 4º C with agitation; immunocomplexes were pelleted by centrifugation in a microfuge and washed 3 times, twice with the

extraction buffer and one final time with 100 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% NP40. Bound proteins were eluted and prepared for immunoblotting with anti-GEM rabbit polyclonal antibody.

Nuclear DNA content analysis

The flow cytometric analysis of leaf and cotyledon nuclei were performed as previously described (Castellano et al., 2004).

Results and Discussion

Plants show a unique tolerance to changes in the level of cell cycle and DNA replication proteins compared to other higher organisms. Moreover, plants with unscheduled levels of pre-RC components do not turn on transformation or oncogenic pathways as animal cells do, making them an interesting model for understanding licensing mechanisms. The ways in which a cell controls the levels of the pre-RC components during the cell cycle is extremely diverse among eukaryotes, in spite of the high conservation of genes. In this work, we have defined the functional domain organization of Cdt1 and our study contributes to enlighten the pathways used by plants to control cell cycle progression and the role of CDT1 as a target for controlling the licensing of DNA replication origins and the switch to the endocycle program.

The N terminus of CDT1 is required for DNA-binding activity

A detailed account of the major landmarks of human Cdt1 is shown on Fig. 1a. These are several Cdk phosphorylation sites, the cyclin-binding (Cy) motif and the PCNA-interacting protein (PIP) motif, all close to the N-terminus. It also contains a central and a C-terminal conserved region as well as a PEST sequence motif. The overall aminoacid sequence homology between human and Arabidopsis CDT1 proteins is relatively low. However, most of the structural features are conserved, except for the absence of a PIP box in Arabidopsis CDT1 (see also below). To define domains and features relevant for various aspects of CDT1 function we generated two truncated versions, as indicated in Fig. 1a, which were used throughout this study.

On the first place we observed that recombinant GST-CDT1a was able to bind to DNA in electrophoretic mobility shift assays, regardless the length, sequence and conformation (single stranded or double stranded) of the probe used (Fig. 1b). Using the truncated versions of AtCDT1 (Fig. 1a) to identify the domain responsible

for the DNA binding activity, we found that the N terminal domain of AtCDT1 is necessary for this function (Fig. 1c). It is interesting to note that mouse Cdt1 also has its DNA binding activity located in the N terminal region of the protein, the region with the lower degree of conservation between all organisms. Thus, it seems that the function, but not the sequence, has remained conserved during evolution.

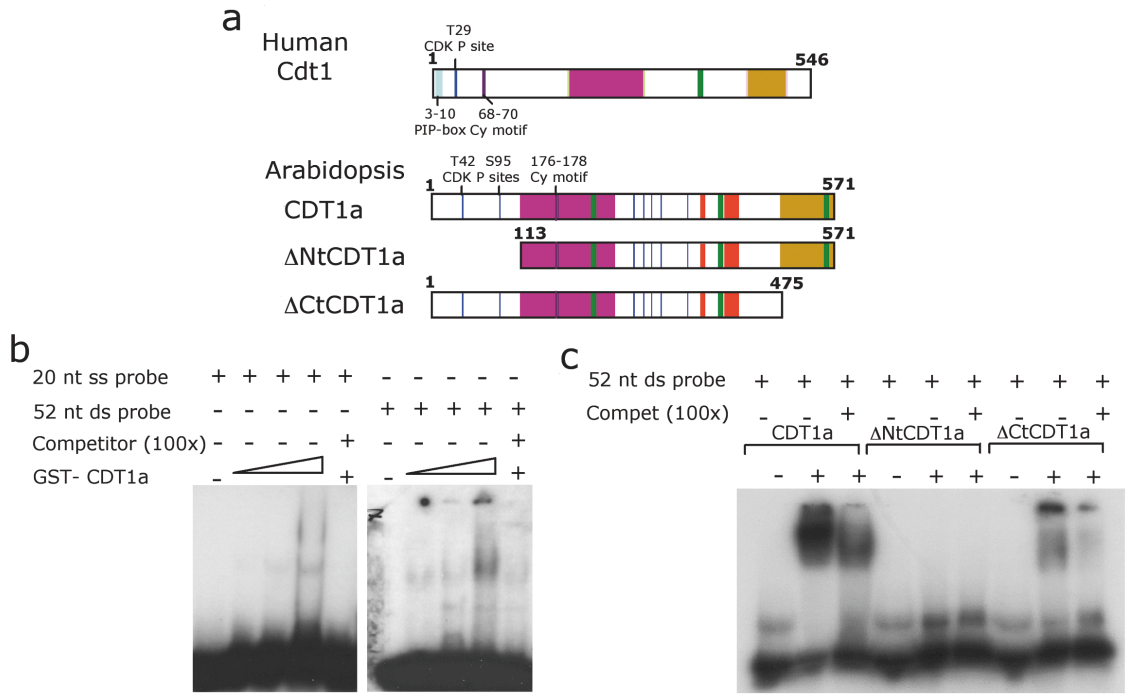

Figure 1: Electrophoretic mobility shift assays of AtCDT1a and truncated versions.
a) Scheme of HsCDT1 and AtCDT1a proteins (representing the central domain, C-terminal domain, PIP box, CDK phosphorylation sites, cyclin binding motif (Cy motif), PEST sequences and destruction boxes). The truncated versions of AtCDT1a used in c are also represented.
b) Various amounts of Arabidopsis GST-CDT1a were incubated with radiolabeled oligonucleotides of different lengths, sequence and conformations as indicated, and the DNA-protein complexes were fractionated by electrophoresis in 4% polyacrylamide gels.
c) CDT1a and the N and C terminal truncated versions fused to GST were incubated with radiolabeled 52 nucleotide double stranded oligonucleotides. Note that the N terminal domain of CDT1 is necessary for its DNA binding activity.

Interaction with CDC6

The function of Cdt1 as a licensing factor requires the ability to bind to DNA replication origins sequentially after ORC and Cdc6. *Schizosaccharomyces pombe* Cdt1 has been shown to associate with Cdc18 (Nishitani et al., 2000), the Cdc6 homologue. However, although human Cdc6 and Cdt1 interact specifically (Cook et al., 2004), the domain in Cdt1 responsible for this interaction has not been defined.

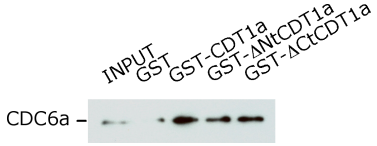

Figure 2: CDT1 interaction with CDC6.
In vitro pull-down assays between His-tagged CDC6 and GST-CDT1 fusion proteins and the truncated versions described in Fig. 1a.

We found that binding of Arabidopsis CDC6 to the truncated versions of CDT1 was undistinguishable from that with the entire protein (Fig. 2), at least in *in vitro* pull-down experiments. This suggests that the central domain, conserved between human and plant Cdt1, is sufficient for Cdc6 binding.

CDT1 interacts with SKP2a

In humans, SCF^{Skp2} ubiquitin ligase targets Cdt1 for degradation after Cdk/Cyclin complexes phosphorylate it (Li et al., 2003). Cdt1 phosphorylation is dependent on the cyclin-binding (Cy) motif within Cdt1 and the phosphorylation on threonine 29 is required for interaction with Skp2 (Liu et al., 2004; Sugimoto et al., 2004). Since SCF^{Skp2}-mediated degradation of Cdt1 is not conserved in non-mammalian species, it is important to establish whether this is also the case in plants. Surprisingly, pull down experiments revealed a clear interaction between Arabidopsis CDT1a and SKP2a proteins (Fig. 3a). CDT1 has already been shown to be a CDK phosphorylation target in Arabidopsis (Castellano et al., 2004), and CDT1a-SKP2a interaction was dependent on phosphorylation, since it was only detected when the interaction medium was supplemented with extra ATP, creatine phosphate and creatine kinase (Fig. 3a).

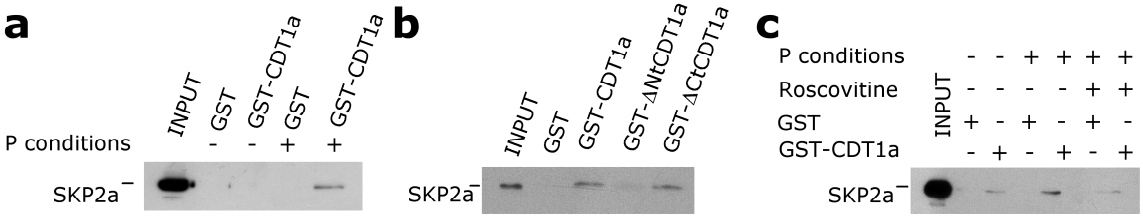

Figure 3: SCF^{Skp2} mediated degradation of Arabidopsis CDT1a
a) *In vivo* pull-down assay, showing interaction of GST-CDT1 with SKP2a from $SKP2a^{OE}$ plant extracts, only when 5mM ATP, 5mM creatine phosphate, 10 U/ml creatine kinase and 0.05 μg/ml ubiquitin were added to the medium prior to the incubation with the recombinant protein (P conditions).
b) Same experiment as in a) but using the truncated versions of CDT1, as described in Figure 1a.
c) CDT1a-SKP2a interaction is diminished when CDKs are inhibited in the extracts by addition of roscovitine.

Furthermore, the CDT1-SKP2a interaction requires the N terminal region of CDT1 (Fig. 3b), where two Cdk phosphorylation consensus sites, conserved in human Cdt1, are located (Fig. 1a). Consistent with a requirement of CDT1 phosphorylation for efficient binding to SKP2a, CDT1a-SKP2a interaction was partially but reproducibly reduced when the extract was treated with the Cdk inhibitor roscovitine (Fig. 3c). Together, we conclude that CDT1a-SKP2a interaction requires phosphorylation and the CDT1 N-terminal domain, suggesting that CDT1 is targeted for proteolysis by a SKP2a-containing SCF complex. This is consistent with the reduction in ploidy levels of $SKP2a^{OE}$ plants (del Pozo et al., 2006) and points

to CDT1 as a key player in controlling the endocycle program.

CDT1 is degraded by SKP2 and CUL4-dependent pathways

CUL4-DDB1 ubiquitin ligase has been proposed to be an ancient mechanism conserved from fission yeast to humans for Cdt1 degradation in response to S-phase entry and DNA damage (Kim and Kipreos, 2007). The degradation of Cdt1 by Cul4 requires the interaction between Cdt1 and PCNA (Arias and Walter, 2006; Hu and Xiong, 2006; Nishitani et al., 2006; Senga et al., 2006). Cdt1 binds PCNA through a PCNA-interacting protein (PIP) box motif in the Cdt1 N terminus in S-phase of the cell cycle and as a response to UV irradiation. It is surprising that all metazoan Cdt1 proteins analyzed show a PIP box in the N terminus of their sequence (Arias and Walter, 2006), while neither Arabidopsis nor any other plant do (Fig. 4a). We tested whether Arabidopsis CDT1 could bind *At*PCNA from Arabidopsis plant extracts, perhaps through a degenerated and unrecognizable PIP box. As expected from the CDT1 sequence, under our experimental conditions, Arabidopsis CDT1 did not interact with PCNA, although PCNA was able to recognize and bind to Xenopus Cdt1 (Fig. 4b), used here as a control.

We also tested whether the mechanisms for Cdt1 degradation that operates in human cells after UV irradiation and dependent on Cdt1-PCNA interaction (Arias and Walter, 2006; Higa et al., 2006; Hu and Xiong, 2006; Nishitani et al., 2006; Senga et al., 2006) was maintained in Arabidopsis in a PCNA independent manner. Time course analysis after UV irradiation showed a decrease in CDT1 levels 15 minutes after the damage had been produced (Fig. 4c), suggesting that the global mechanism is conserved.

To establish the relevance of SKP2 and CUL4 dependent pathways in controlling CDT1 levels, we used Arabidopsis plants carrying mutations in the *SKP2* and *CUL4* genes. We generated the double *skp2a,skp2b* and the triple *cul4,skp2a,skp2b* mutants that express a FLAG-tagged CDT1a protein in an inducible manner. We found that CDT1 is partially stabilized in *skp2a,skp2b* mutants and only in the triple *cul4,skp2a,skp2b* mutant the levels of CDT1 were independent of the proteasome inhibitor MG132 (Fig. 4d). This strongly suggests that two different mechanisms exist to control CDT1 degradation by the proteasome, involving two different ubiquitin ligase complexes containing SKP2 and CUL4, and that no other proteasome degradation mechanism exists for CDT1 protein.

It is worth noting that the first mechanism seems to be shared by humans and

plants, whereas the second one, though conserved in yeast, mammals and plants, bears an important difference in plants, where it is PCNA independent.

Increased levels of CDT1 induce extra endocycles during leaf development (Castellano et al., 2004). It has been published that defects in chromatin assembly during the S-phase and DNA damage ultimately lead to mitotic arrest and trigger the endocycle program (Ramirez-Parra and Gutierrez, 2007), however *CDT1* overexpression does not lead to the activation of DNA damage pathways involving *ku70*, *BRCA1* or *PARP1*, since their expression levels are not altered in CDT1^{OE} plants (data not shown), suggesting that CDT1 is a key target directly involved in the switch to the endocycle program.

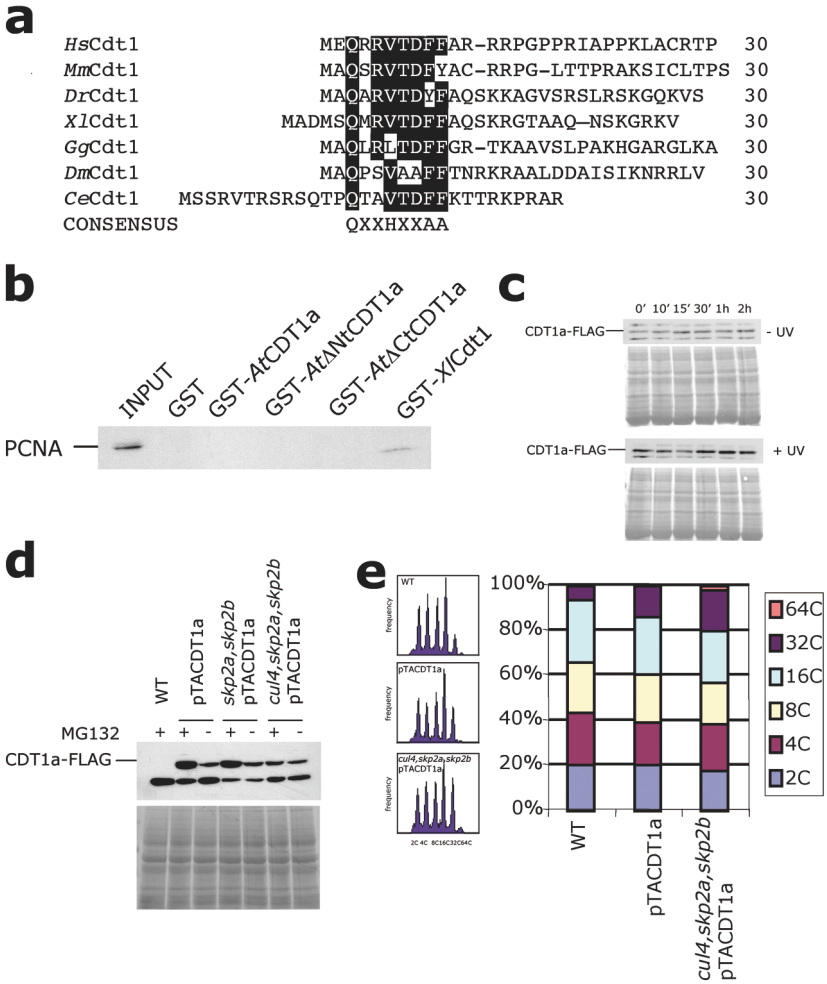

Figure 4: Arabidopsis CDT1 degradation mechanisms includes CUL4 ubiquitin ligase complex.
a) PIP box in the N terminal region of metazoans Cdt1 sequence
b) Pull-down assays between recombinant Cdt1 proteins and a WT plants extract. Arabidopsis CDT1 does not bind PCNA while Xenopus Cdt1 (XlCDT1) is able to do so.
c) CDT1 dynamics in plants constitutively expressing CDT1a-FLAG after UV irradiation.
d) CDT1 expression in CDT1^{OE}, *skp2a,skp2b,*CDT1^{OE} and *cul4,skp2a,skp2b,*CDT1^{OE} plants ± MG132.
e) DNA content analysis in cotyledons of 14 das plants, 10 days after induction.

It is interesting that the abrogation of the two mechanisms for CDT1 targeting to degradation is not enough to produce an increase in nuclear DNA content of leaf or cotyledon cells (data not shown). However, in the triple *cul4,skp2a,skp2b* mutant, the induction of CDT1 expression causes a polyploidy effect even stronger than that observed in the WT background (Fig 4e). These results suggest that although CDT1 protein is stabilized in *cul4,skp2a,skp2b* mutants, the obtained levels of the protein are not sufficient to trigger the switch to the endocycle program, and that a threshold must exist, over which CDT1 stabilization can even induce extra endocycles. It is possible that a third redundant mechanism of control exists, that *At*CDT1 is inhibited by the interaction with another factor, as seen in metazoans for geminin, or that it is exported out of the nucleus during S phase once the licensing of the origins has occurred, so future studies will be focused on the study of these possibilities.

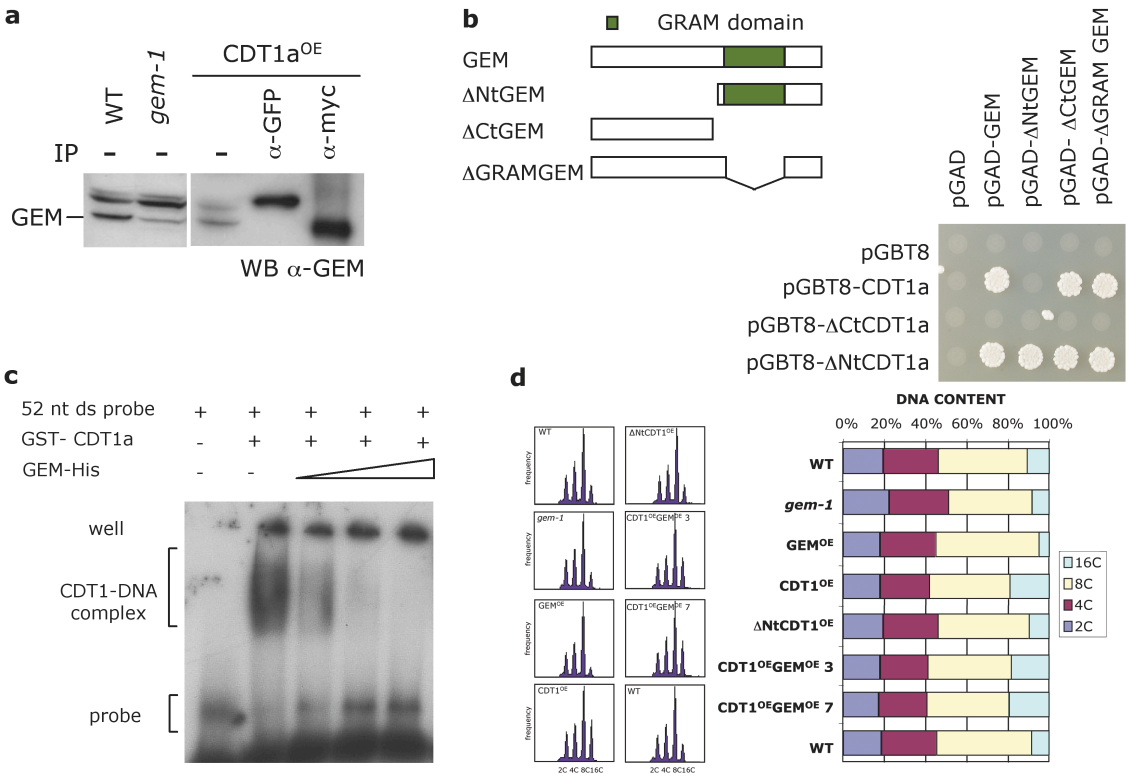

Figure 5: GEM function in replication as a CDT1 inhibitor
a) Coimmunoprecipitation of CDT1 and GEM in CDT1^{OE} plants.
b) Yeast two hybrid analysis of the domains responsible for CDT1a and GEM interaction. The GEM truncated versions used for the analysis appear represented in the scheme. The truncated versions of CDT1a used here are described in Figure 1a.
c) Electrophoretic mobility shift assay showing that GEM disrupts CDT1-DNA binding.
d) Cytometry analysis of the DNA content of the nuclei of leaves 1 and 2 from 22 days-after-sowing plants.

Interaction with GEM

The last redundant mechanism for Cdt1 availability control present in metazoans is the Cdt1-interacting protein geminin that inhibits Cdt1 function (McGarry and

Kirschner, 1998). Considering that geminin homologues have not been found neither in yeast nor in plants, it is commonly thought that it may have evolved in contexts other than regulation of DNA replication and thereafter have become adapted for roles in Cdt1 regulation (Fujita, 2006). Nevertheless, we have recently identified a CDT1-interacting protein, GEM, in Arabidopsis (Caro et al., 2007) and we found that all other plants whose genome is completely or partially sequenced contain GEM homologues (Caro and Gutierrez, 2007). GEM shows no sequence similarity with metazoan geminin homologues and has a very poor probability of folding as a coiled coil, the main structure involved in mouse and human Cdt1-geminin interactions (Lee et al., 2004). In spite that GEM and geminin are unrelated proteins, we investigated whether GEM may have a role in regulating CDT1 function in endoreplication control. Immunoprecipitation assays showed that GEM binds CDT1 *in vivo* (Fig. 5a). We used yeast two hybrid assays to map the domains responsible for this interaction, and found that the N terminal region of GEM and the C terminal region of Cdt1 are necessary for the interaction (Fig. 5b).

The CDT1 domain responsible for GEM binding is not the same as the domain responsible for human Cdt1 interaction with geminin, supporting that there is no conservation in GEM-CDT1 and geminin-Cdt1 interactions. However, GEM interaction with CDT1 produces an inhibitory effect on the CDT1 DNA binding activity (Fig. 5c) similar to that shown for mouse geminin binding to Cdt1 (Yanagi et al., 2002). Whether this effect is only observed in *in vitro* experiments or can be applied to endogenous systems remains a question.

To unveil the possible function of GEM in endoreplication control, we analyzed DNA content in plants with altered levels of GEM. We found that *gem-1* and GEM^{OE} plants showed little effect, if any, on ploidy levels during leaf development (Fig. 5d). To test whether *GEM* overexpression can suppress the ploidy increase described for $CDT1^{OE}$ plants (Castellano et al., 2004), we generated double $CDT1^{OE}GEM^{OE}$ plants, and confirmed by western blot that both proteins were co-expressed (not shown). As shown in Fig. 5d, the ploidy profile of the double overexpressor was undistinguishable from that of $CDT1^{OE}$ plants, indicating that an excess of GEM was not able to counteract the stimulation of endocycle mediated by an excess of CDT1. Further studies, still in process, are necessary to discard the participation of other redundant mechanisms, independent of GEM, in CDT1-mediated endoreplication control.

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Discussion

Organs are specialized tissues used for specialized physiology and environmental adaptation. Organogenesis involves cell proliferation followed by complex determination and differentiation events that are finely controlled in time and space. The first step in organogenesis is the specification of a primordium – the establishment of a group of founder cells committed to form an organ. Secondly, the cells in the primordium proliferate to an appropriate degree, and positional information is generated within the developing tissue, leading to distinct cellular identities in different regions of the organ. Finally, precursor cells undergo cell cycle exit at the correct time and location, and differentiate as cell types specialized for their context. Thus, organogenesis requires a delicate balance between cell proliferation, specification and differentiation (Slack, 2005).

In contrast to animals, organogenesis in plants is a post-embryonic process that requires developmentally programmed reversion of sets of cells from differentiated states to a pluripotent state followed by regulated proliferation and progression through distinct differentiation patterns (Ramirez-Parra et al., 2005). This remarkable fact relies on the existence of stem cell niches, e.g. in the shoot and root apical meristems (Nakajima and Benfey, 2002; Weigel and Jurgens, 2002) that continuously provide new cells that eventually follow specific differentiation pathways.

The control of cell proliferation during organogenesis plays an important role in initiation, growth, and acquisition of the intrinsic size of organs in higher plants. Although plant growth is influenced greatly by external environmental factors, it appears that the intrinsic size of plant organs is determined by internal developmental factors. The total cell number of an organ is determined by the number of divisions of undifferentiated meristematic cells. During development, cells lose meristematic competence and withdraw from the cell cycle. Thus, the maintenance of meristematic competence of cells is a key mechanism that mediates organ growth and cell proliferation by defining total cell number and thereby the size of plant organs (Mizukami and Fischer, 2000). Cell identity acquirement requires differential transcription in different sets of cells. Exploring the regulation of gene transcription is necessary for a detailed understanding of the molecular basis of development.

Pattern formation in animal organogenesis

Numerous cell divisions have to be regulated on the path from a unicellular embryo, the zygote, to the multicellular structures of a mature being. Numerous functions, specializations and cellular identities have to be generated, in order to form a complex and mature animal. Numerous mechanisms have to control the correct assignment and acquisition of cellular fates, as well as the right timing and allocation of cells. Therefore, a strict coordination has to occur between embryonic patterning and the cell cycle. In animals, different protein families with this coordination role have already been described, here we will discuss briefly homeobox, hedgehog, myc and geminin.

Determination of regional identity and specification of the metazoan body plan are achieved through the localized activities of homeodomain proteins. A **homeobox** is a DNA sequence found within genes that are involved in the regulation of development and morphogenesis of animals (Gehring, 2007), fungi (Iimura and Pourquie, 2007), and also plants (Vollbrecht et al., 1991), indicating that molecular mechanisms underlying development may be much more universal than previously suspected (Gehring, 2007). While certainly not the only developmental control genes, homeobox genes have been shown to play crucial roles from the earliest steps in embryogenesis - such as setting up an anterior-posterior gradient in the egg of Drosophila - to the very latest steps in cell differentiation - such as the differentiation of neurons in *C. elegans* (Gehring, 2007).

But homeobox genes are not the only important orchestrators of development. The **hedgehog** family, including Sonic hedgehog (Shh), is the most well-known morphogen involved in the developmental pattern formation of various organs, such as face, limbs, and skin appendages. In neural development, Hedgehog proteins function in directing neural progenitors to acquire specific cell identities and in addition, have a mitogenic role in controlling the proliferation of neural progenitors and in the maintenance of adult stem cells (Fuccillo et al., 2006).

Myc genes, emerged almost 30 years ago as members of the MYC/MAX/MAD network of the basic region/helix-loop-helix/leucine zipper (bHLHZ) domain transcriptional regulators, have been implicated in human cells in the genesis of human malignancies. In addition the Myc promoter is targeted by multiple signal transduction cascades that are deregulated in cancer cells and contribute to enhanced Myc expression. The deregulation of Myc target genes produces effects on cell behavior and the inability for a cell to exit the cell cycle and enter a

quiescent state or to differentiate in response to appropriate signals (Vervoorts et al., 2006).

Geminin is a protein with a dual role during metazoan development. Geminin was originally identified as a DNA replication inhibitor, notwithstanding, recent experimental works show that Geminin is involved both in the regulation of proiferation by arresting Cdt1, and in the regulation of Hox- and Polycomb-dependent embryonic patterning. Through its participation in both multiprotein machineries, Geminin is considered a key element in the coordination of cell cycle and developmental control (Luo and Kessel, 2004).

Pattern formation in plant organogenesis

In the past decade, many efforts have been done to genetically dissect the mechanisms underlying pattern formation in plant embryos as well as later in development. The adult body of vascular plants is the result of meristematic activity. In plant meristems, dividing cells interpret positional information and translate it into patterned cell differentiation. Surgical studies and clonal analysis have revealed indirectly that cells in meristems have no predictable destiny and that position is likely to play a role in the acquisition of cell identity. In contrast to animal systems, it is positional control that is most important in the determination of cell fate. The analysis of cell fate depending on position can be easily determined in a simple and structured system like the root meristem.

Mutations in the ***HOBBIT*** (*HBT*) gene interfere with root formation, specifically with the specifying of the hypophyseal cell, the progenitor cell for the QC and columella, and the proper formation of a lateral root cap (Scheres et al., 1996). *hbt* mutants lack meristem activity and the QC, columella and lateral root cap do not differentiate (Willemsen et al., 1998). The *HBT* gene encodes a homologue of a subunit of the yeast Anaphase Promoting Complex and may couple cell division to cell differentiation by regulating cell cycle progression in the meristem or by restricting the response to differentiation cues, such as auxin, of dividing cells (Blilou et al., 2002).

In ***tornado*** (*trn1* and *trn2*) mutants the formative divisions of the epidermal/lateral root cap initial are defective. Cells with lateral root cap fate are found in the cell layer which normally contains epidermal cells (Cnops et al., 2000). This indicates that TRN1 and TRN2 are required for correct cell specification in the outermost layer of cells and that the role of these gene products is to repress lateral root cap

fate in cells in the epidermal location.

Mutants have been identified that result in changes in the establishment of the apical-basal (Hemerly et al., 2000; Jenik et al., 2005) and the radial pattern (Scheres et al., 1995).

A mutation in the ***TILTED1*** locus, which encodes the catalytic subunit of DNA polymerase ε of *Arabidopsis thaliana*, causes a lengthening of the cell cycle throughout embryo development and alters cell type patterning of the hypophyseal lineage in the root, leading to a lateral displacement of the root pole from its normal position on top of the suspensor (Jenik et al., 2005), what results in the shoot–root axis of the embryo being at an angle, or tilted, relative to the embryo-suspensor axis. Treatment of preglobular and early globular stages, but not later stage, embryos with the DNA polymerase inhibitor aphidicolin leads to a similar phenotype. These results uncover an interaction between the cell cycle and the processes that determine cell fate during plant embryogenesis.

Indeed, the reduction of the frequency of cell division exclusively during embryo development by the expression of a dominant ***cdc2a*** mutant leads to affected embryo formation. Mutant seedlings display a range of distortions on the apical–basal embryo pattern and in most seedlings, initiation of root formation does not occur. In few seedlings, traces of the embryonic root are present, whereas in others, the main root starts to develop, but without growing any further, thus suggesting that they lack a functional meristem. When the roots manage to continue growing, the developing root contains the major cell types. Because the *At*2S2 promoter that drives mutant *cdc2a* expression becomes active at late-heart stage, when the major embryo structures and the main tissue types (protoderm, ground and vascular tissues) can already be distinguished, it seems that apical–basal pattern elaboration depends on cell division, despite all pattern elements and cell types being produced correctly.

The radial organization of the root is generated by stereotyped division of initial cells and subsequent acquisition of cell fate. In a transverse root section, there are four radially symmetric layers (epidermis, cortex, endodermis, pericycle) that surround the bilaterally symmetric vascular tissue (Benfey and Scheres, 2000). Mutations that disrupt the radial pattern have been useful in identifying genes that play important roles in establishing and maintaining it (Scheres et al., 1995). In the ***scarecrow*** (*scr)* and ***short-root*** (*shr*) mutants, instead of cortex and endodermis,

a single mutant layer between the epidermis and the vasculature develops (van den Berg et al., 1995). Analysis of tissue-specific markers revealed that the mutant layer is specified differently in the two mutants. In *scr*, markers for both cortex and endodermis are present in the mutant layer, indicating that SCR is required for the periclinal division of the initial cell, but does not play a role in cell specification (Di Laurenzio et al., 1996). In *shr*, only markers for cortex are found, indicating that SHR is required for both the longitudinal cell division of the initial as well as endodermal cell specification (Di Laurenzio et al., 1996). Further studies showed that SHR protein is able to translocate from the vasculature to cells of the adjacent layer (Nakajima et al., 2001), suggesting that SHR acts both as an activator of cell division and an inducer of cell fate as well as a positional signal.

The root epidermis is composed of two cell types whose identity is regulated by positional information: trichoblasts and atrichoblasts. Laser ablation experiments and clonal analysis indicates that positional cues direct cell identity and there is some evidence that these positional cues are located in the cell wall (Berger et al., 1998a). Once hair cells have been specified, the hairs are initiated in a polar localization dependent on an ethylene and auxin pathway (Schiefelbein and Benfey, 1991). Trichoblasts are smaller than atrichoblasts throughout their development in the meristem, elongation and differentiation zones (Dolan et al., 1993; Galway et al., 1994). For cells in the trichoblast position to be shorter than those in the atrichoblast position the rates of cell division must be greater in the trichoblasts than atrichoblasts. Therefore upon switching positions, the cell division parameters are altered, resulting in the formation of cells with the appropriate dimensions. This indicates that relative cell size and cell division rates are strictly regulated in the epidermis (Berger et al., 1998b).

Transparent testa glabra 1 (*ttg1*) mutants develop hairs on every cell in the epidermis but longitudinal divisions take place in both atrichoblast and trichoblast positions, indicating that TTG1 is also required for the suppression of longitudinal divisions in the atrichoblasts (Berger et al., 1998b). On the other hand every epidermal cell in ***glabra2*** (*gl2*) mutants develop hairs but no longitudinal divisions occur in the atrichoblasts, indicating that the specification of cell fate and longitudinal divisions can be uncoupled. Together these data indicate that a subset of the genes regulating cell specification also regulate the plane of cell division in the epidermis (Scheres, 2002).

Mutants in the *GL2-expression-modulator* (***GEM***) gene have shown problems in cell

fate determination, since epidermal cells lose its dependence on position relative to the cortex (Caro et al., 2007). In addition, GEM is a general repressor of cell division in the three division planes. However, it is interesting that *GEM* mutation effect on cell division is present in all kinds of cells studied, including the columella stem cells, endodermis, cortex and epidermis, while its effect on cell fate is specific to the epidermis, the only layer where two different cell types are specified. The mechanism by which GEM controls epidermal cell fate is far away from clear, but at least we have some hints that point to GEM interaction with chromatin remodeling pathways to control expression of the patterning genes *GL2* and *CPC* through controlling the acetylation and methylation status of H3K9 at their promoters. Nevertheless, GEM's action in cell cycle control remains unknown, since GEM involvement in cell cycle progression control through its interaction with CDT1 is not consistent with CDT1 having little or no effect on transversal divisions and meristem size establishment within the developing root.

All these results are surprisingly reminiscent of the functions reported for geminin in animals as a regulator of the proliferation-differentiation balance by the integration of cell cycle and transcriptional controls during organogenesis (Kroll, 2007). Although GEM and geminin possess an opposite expression pattern relative to the proliferative status of the cell, they are functionally analogous in regards to their roles in cell division and transcriptional control of genes regulating the transition of proliferating cells to a differentiating state through chromatin dynamics (Caro and Gutierrez, 2007).

Acquisition of the multicellular level of complexity is associated with the development of cellular structures specialized in highly specific functions as a result of the need to communicate, cooperate and compete. Thus, the coordination of cell proliferation, cell fate decisions and cell differentiation required for organ formation is at the basis of multicellularity. Multicellular organization emerged from different unicellular ancestors at independent times in animals and plants, and the functional convergence between GEM and geminin is remarkable despite the fundamental differences in organogenesis, body plan structure and developmental cues between plants and animals.

Endoreplication control

An intriguing analogy between GEM and geminin refers to their ability to interact with Cdt1. Cdt1 is a licensing factor for DNA replication, conserved among all eukaryotes and whose function is tightly controlled to maintain genome integrity. In

most eukaryotic organisms, Cdt1 is a key target over which the main control pathways are established and it has been demonstrated that pre-RC regulation during the cell cycle is extremely diverse among eukaryotes, in spite of a high conservation of the proteins involved (Kim and Kipreos, 2007). Some of the mechanisms that have evolved in the different organisms to regulate Cdt1 function include changes in the subcellular localization, modulation of DNA binding activity, degradation and direct inhibition.

In metazoans, geminin inhibits re-replication during S and G2 phase by binding the essential replication protein Cdt1. This role in preventing replication abnormalities of geminin is also required for entry into mitosis (Saxena and Dutta, 2005). Plant genomes bear no geminin homologue (Caro and Gutierrez, 2007) and seem to lack the Cdt1 inhibition mechanism present in metazoans (this Thesis, Chapter 4). However, there exists GEM, a CDT1-interactor protein with functional analogies to geminin, and its role in cell cycle progression remains unknown. Cdt1 overexpression can induce re-replication in mammalian cells (Vaziri et al., 2003) although studies of the consequences of Cdt1 deregulation in whole organisms are scarce. Strong mutations in Dup (the Drosophila Cdt1 homologue) cause embryonic lethality (Whittaker et al., 2000) and stabilization of *C. elegans* Cdt1 in *cul4* mutants causes massive DNA re-replication and lethality among the progeny (Zhong et al., 2003). Genetic ablation of geminin in mouse causes premature endoreduplication resulting in mbryonic lethality (Gonzalez et al., 2006). Deregulation of Arabidopsis CDT1 showed that plant growth is compatible with moderately, though not highly, increased levels of *At*CDT1a (Castellano et al., 2004). This is not strange, since plants have proven intriguingly tolerant to changes in the level of cell cycle and DNA replication proteins (reviewed in Gutierrez et al., 2002; Gutierrez, 2005; Inzé and De Veylder, 2006). CDT1 overexpression caused that extra endocycles were triggered in cells programmed to undergo differentiation-associated endoreplication rounds (Castellano et al., 2004). Within the endoreplication cycle, the duplicated genetic material does not end up in two daughter cells, but instead, remains in the mother cell. In plants, it is a common form of the cell cycle that has been related to processes such as cell differentiation, cell expansion, metabolic activity and fitness for survival (Sugimoto-Shirasu and Roberts, 2003; Barow, 2006; Caro et al., 2008). Our data suggests that CDT1 is a major target in endoreplication control, and that many different strategies exist to control this function.

In Chapter 4 of this Thesis we have shown that Arabidopsis CDT1 is subjected to

proreasome degradation by SCF^{Skp2} similarly to what happens in human cells. The ancient degradation pathway present from yeast to mammals that involves ubiquitination by a Cul4-based complex is also conserved in Arabidopsis, though it seems to be independent from CDT1-PCNA binding. However, other redundant strategies might exist that still remain unknown, since abrogation of the SKP2- and CUL4-mediated degradation systems is not enough to produce an over-replication phenotype in developing Arabidopsis plants. Future studies should aim at their discovery.

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Conclusions

The main conclusions achieved after this Thesis work are:

1. **GEM controls epidermal cell fate specification.** GEM is a novel CDT1-interactor protein that plays a dual role in plant development. It regulates epidermal cell fate acquisition by its interaction with TTG1 as part of the network regulating *GL2* and *CPC* expression through the control of H3K9 acetylation and methylation status at patterning genes promoters.

2. **GEM represses epidermis and cortex anticlinal longitudinal cell division.** GEM also acts as a repressor of the proliferation potential of the cells in the epidermis and cortex layers of Arabidopsis root meristems. Thus, GEM seems to be a crucial component for root architecture, providing a link between cell division, fate and differentiation during Arabidopsis root development.

3. **GEM and geminin share interesting functions as coordinators of proliferation and differentitation during organogenesis.** The general strategies in controlling the cell division/cell fate balance during development of the multicellular major lineages, plants and animals, follow similar strategies, though using unrelated proteins, GEM and geminin, respectively. GEM and geminin appear to share a number of functional properties: ability to bind Cdt1, participation in bHLH-containing transcriptional complexes, repression of homeobox genes and genes that function at the interface between progenitor undifferentiated cells and differentiating cells, capacity to modulate histone marks and/or interact with chromatin remodeling complexes, and regulation of the cell division potential.

4. **CDT1 is a key target of over-replication control.** Cdt1, a licensing factor for DNA replication, is a key target in Arabidopsis endoreplication control. CDT1 is targeted to proteasome degradation by SCF^{Skp2} and a Cul4-based complex. The first strategy is common to humans and the second is present from yeast to mammals, though in Arabidopsis does not seem to require CDT1-PCNA binding.

5. **Endoreplication control by CDT1 is independent of GEM.** The CDT1 inhibition mechanism present in metazoans, however, does not seem to happen in plant cells, at least as an over-replication control pathway. Other redundant strategies that still remain unknown might exist, since an abrogation of the degradation systems is not enough to produce over-endoreplication in developing Arabidopsis plants.

6. **GEM represses the change in the division plane during root development.** GEM is a general repressor of cell division in the Arabidopsis root meristem. It represses transversal cell division and consequently, reduces meristem size. GEM also specifically inhibits the change in the division plane necessary to generate the longitudinal divisions (anticlinal and periclinal) responsible for the increase of growth of the root.

7. **GEM represses stem cell division.** GEM is involved in cell division repression in columella stem cells and QC cells, confirming GEM's role in assuring a correct organ pattern and development.

Conclusiones

Las principales conclusiones de la presente tesis son:

1. **GEM controla la toma de decisiones de identidad de las células epidérmicas.** GEM es una nueva proteína que interacciona con CDT1 y que posee una función dual en el desarrollo vegetal. Regula la toma de decisiones de identidad celular epidérmica mediante su interacción con TTG1 y como parte del complejo que regula *GL2* y *CPC* mediate el control del estado de la acetilación y la metilación de la H3K9 en los promotores de los genes de formación de patrón.

2. **GEM reprime las divisiones anticlinales longitudinales de la epidermis y el cortex.** GEM también actúa como un represor del potencial proliferativo de las células de la epidermis y el cortex en los meristemos radiculares de Arabidopsis. Por lo tanto, GEM es un componente crucial para la arquitectura radicular ya que es un coordinador de la división celular, la identidad y la diferenciación durante el desarrollo de la raíz de Arabidopsis.

3. **GEM y geminina comparten interesantes funciones como coordinadores de la proliferación y la diferenciación durante el proceso de organogénesis.** Las estrategias generales que controlan el balance división/toma de identidad celular durante el desarrollo de los principales linajes multicelulares, plantas y animales, siguen estrategias similares, aunque usan proteínas no relacionadas, GEM y geminina, respectivamente. GEM y geminina comparten cierto número de propiedades funcionales: interacción con Cdt1, participación en complejos transcripcionales que contienen factores bHLH, represión de genes homeobox y genes que funcionan en la interfase entre células progenitoras indiferenciadas y células en diferenciación, capacidad para modular marcas de histonas y/o interaccionar con complejos remodeladores de la cromatina, y regulación del potencial de división celular.

4. **CDT1 es un objetivo clave para el control de la endorrepliación.** CDT1, un factor de licenciamiento de la replicación de DNA, es una diana clave para el control de a endoreplicación en Arabidopsis. CDT1 sufre degradación por el proteosoma mediante SCF^{Skp2} y un complejo basado en Cul4. La primera es una estrategia común con humanos y la segunda está

presente desde levaduras hasta mamíferos, aunque en Arabidopsis no parece requerir la interacción CDT1/PCNA.

5. **La ruta de control de la endorreplicación por CDT1 es independiente de GEM.** El mecanismo de inhibición de Cdt1 presente en metazoos no parece operar en células vegetales, al menos como vía de control de la endoreplicación, pero deben existir otras estrategias redundantes aún desconocidas, ya que la abrogación de los sistemas de degradación no es suficiente para producir endoreplicación extra en plantas de Arabidopsis en desarrollo.

6. **GEM reprime el cambio en el plano de división durante el desarrollo radicular.** GEM es un represor general de la división celular en el meristemo radicular de Arabidopsis. GEM reprime las divisiones transversales y, por lo tanto regula negativamente el tamaño de meristemo, e inhibe específicamente el cambio de plano de división necesario para la generación de divisiones longitudinales (anticlinales y periclinales) responsables del incremento en grosor de la raíz.

7. **GEM reprime la división de las células madre.** GEM también está involucrada en la represión de la división celular en las células madre de la columela y el centro quiescente, lo que confirma la implicación de GEM en el control de la formación del patrón correcto de órgano y desarrollo.